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<p>13. ABSTRACT <i>(Maximum 200 words)</i></p> <p>Oncoproteins of the Myc family are important regulators of cellular proliferation, differentiation, transformation, and apoptosis. Overwhelming evidence suggests the transcription activation domain, or TAD, of Myc is responsible for mediating all of Myc's functions <i>in vivo</i>. In an attempt to more clearly define regions of the amino terminus important to Myc activity, deletion mutagenesis of the v-Myc TAD was performed. Examination of the truncated Myc proteins by reporter gene assays identified a region within the v-Myc amino terminus that acts as a negative regulatory domain. A yeast two-hybrid screen also was performed using a highly conserved domain of the v-Myc TAD called <u>Myc homology region II</u>, or MHR II. Analysis of the 22 positive clones obtained indicated that 8 represented overlapping isolates of the cDNA encoding the OS-9 protein. In vitro binding experiments demonstrate OS-9 specifically interacts with regions of the v-Myc TAD that contain MHR II. To determine the functional consequences of the Myc/OS-9 interaction, OS-9 was overexpressed in cells. Interestingly, co-expression of OS-9 dramatically inhibits transactivation by Myc and enhances transcriptional repression by Myc. More importantly, expression of OS-9 slows the growth of cells stimulated to proliferate by v-Myc overexpression. These data indicate that OS-9 is a novel modulator of v-Myc transcriptional activity, and this modulation has important consequences to the growth of cells.</p>			
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SECTION 1: INTRODUCTION

The *myc* gene was originally identified as the transforming sequence of the avian leukemia virus MC29 (Sheiness *et al.*, 1978). Infection of birds with this virus causes carcinomas, endotheliomas, and sarcomas besides myelocytomatosis, a leukemic condition from which *myc* was named (reviewed in Graf and Beug, 1978). The cellular homolog of *v-myc*, *c-myc*, was first identified in chickens (Vennstrom *et al.*, 1982) and has subsequently been identified and cloned in mammals (humans, mice, rats), amphibians (*Xenopus laevis*), fish (*Brachydanio rerio*), nonvertebrates (the sea star *Asterias vulgaris*) (reviewed in Henriksson and Luscher, 1996), and recently, in the fruit fly (*Drosophila melanogaster*) (Schreiber-Agus *et al.*, 1997). However, a *c-myc* homolog has not been identified to date in the worm (*Caenorhabditis elegans*) or in yeast (*Saccharomyces cerevisiae*).

The v-Myc protein is a 110 kDa fusion protein (Bister *et al.*, 1977) encoded by both viral *gag* sequences and *myc*-specific sequences. v-Myc (Figure 1.1) consists of 459 residues encoded by viral *gag* sequences and 416 amino acids encoded by transduced *c-myc* sequence (Alitalo *et al.*, 1983). v-Myc is a phosphoprotein (Bister *et al.*, 1982) that is located in the nucleus of infected cells (Eisenman *et al.*, 1985). Despite the presence of *gag*-encoded sequences in v-Myc, the Gag residues do not influence the transforming activity of v-Myc (Bister *et al.*, 1987). The v-Myc protein also differs from the c-Myc protein in its ability to transform cells. Primary avian embryo cell cultures of chicken or quail fibroblasts or macrophages are efficiently transformed by expression of v-Myc (Beug *et al.*, 1979). In contrast, c-Myc cannot transform primary rat fibroblasts without cooperation from an activated *ras* oncogene (reviewed in Marcu *et al.*, 1992). c-Myc can, however, transform certain established cell lines independently (reviewed in Cole, 1986). Despite extensive sequence homology, v-Myc and c-Myc are unique proteins which differ in transforming abilities.

myc genes comprise a group of “immediate early response” genes whose expression is activated upon mitogenic stimulation of noncycling, quiescent cells (reviewed in Henriksson and Luscher, 1996). This induction of *myc* expression occurs within two hours of stimulation and does not require *de novo* protein synthesis (reviewed in Marcu *et al.*, 1992). In accordance with its role in cellular proliferation, when human T lymphocytes are cultured in the presence of antisense oligodeoxynucleotides complementary to a region covering the initiation codon of *c-myc* mRNA, c-Myc protein expression is inhibited. The T lymphocytes are also prevented from entering S phase of the cell cycle when stimulated with mitogen (Heikkila *et al.*, 1987). In further support

of Myc's role as a positive inducer of cellular proliferation are studies which examine *myc* expression during the differentiation of certain cells. Mouse erythroleukemia (MEL) cells, when induced to differentiate by treatment with DMSO, show dramatically reduced *c-myc* mRNA levels as compared to *c-myc* mRNA expression in proliferating cells (Lachman and Skoultchi, 1984). Examination of *c-myc* expression in the developing mouse is particularly impressive in the gastrointestinal system, where the levels of c-Myc protein are highest in immature proliferating cells and nearly undetectable in postmitotic differentiated cells (Chin *et al.*, 1995). These studies suggest that Myc proteins are intimately involved in cellular proliferation and are antagonistic to cellular differentiation.

It is not surprising that such a potent inducer of cellular proliferation and repressor of cellular differentiation would be implicated in many human malignancies. For example, misexpression of Myc is involved in approximately 95% of Burkitt's lymphomas. In many cases of Burkitt's lymphoma, a chromosomal rearrangement occurs in which the *c-myc* locus on chromosome 8 is translocated reciprocally to the breakpoint region on the end of chromosome 14. The site of translocation on human chromosome 14 is the immunoglobulin heavy-chain (IgH) gene, which is actively expressed in B lymphocytes. Fusion of *c-myc* to the IgH gene results in continual expression of the *c-myc* gene, leading to tumor development (reviewed in Spencer and Groudine, 1991). Actually, when Burkitt's lymphoma cell lines are analyzed, *c-myc* expression in these lines is typically 20- to 60-fold higher than in normal cultured fibroblasts (Hann and Eisenman, 1984). Besides the chromosomal translocation of *myc* in Burkitt's lymphomas, 65% of these malignancies also involve point mutations in the *c-myc* gene (Bhatia *et al.*, 1993). Interestingly, these point mutations tend to "cluster" in certain critical regions of the Myc protein.

Specifically, mutations in c-Myc repeatedly occur in Myc homology region II (MHR II, see **Figure 1.2**; Yano *et al.*, 1993; Gu *et al.*, 1994), a domain of the Myc protein highly conserved throughout all Myc family members and essential for Myc function. Also, another group of mutations occur often in conserved phosphorylation sites in Myc (Hoang *et al.*, 1995). The recurrence of clustered point mutations in Myc indicate that besides overexpression that occurs due to translocation of the *myc* gene, mutation of *myc* also contributes to malignancy. In addition to the chromosomal translocation of the *c-myc* gene common in Burkitt's lymphoma, amplification of *c-myc* accounts for the formation of as many as 30% of all breast carcinomas and is also responsible for leukemias and carcinomas of the stomach, lung, and colon (reviewed in Dang and Lewis, 1997). Specifically, the *c-myc* gene is amplified in 32% of primary breast carcinomas tested. However, even in many carcinomas with seemingly normal *c-myc* genes, increased *c-myc* mRNA levels were detected. Overall, greater than 70% of invasive ductal

carcinomas studied exhibited high levels of *c-myc* expression (Escot *et al.*, 1986). Also, a recent study by Driouch *et al.* (1997) implicates *c-myc* gene amplification in the genesis of a primary breast tumor as opposed to progression of the tumor due to absence of *c-myc* gene amplification in distant metastases (Driouch *et al.*, 1997). The observations that Myc proteins are important to cell growth and involved in many human tumors prompted many investigators to examine the functions of Myc more closely.

Similar to many other well-characterized transcription factors, the Myc proteins contain a basic helix-loop-helix/leucine zipper (bHLH/LZ) domain in the carboxyl terminus of the protein. The basic helix-loop-helix (Murre *et al.*, 1989) and leucine zipper (Landschulz *et al.*, 1988) motifs mediate protein-protein interactions and facilitate DNA binding by those complexes. Screening of a bacterial expression library with a labeled protein encompassing the bHLH/LZ region of c-Myc resulted in identification of another basic helix-loop-helix/leucine zipper protein termed Max that specifically interacts with Myc (Blackwood and Eisenman, 1991). Upon complex formation between Myc and Max, these heterodimers specifically bind DNA containing the core sequence of -CACGTG-, also called a Myc E box (Blackwell *et al.*, 1990; Kerkhoff *et al.*, 1991). Because many eukaryotic transcriptional regulators contain distinct domains that mediate DNA binding and transcriptional activation (Keegan *et al.*, 1986), and since Myc has been shown to bind specific DNA sequences, the ability of Myc to activate transcription was investigated. As predicted, Myc was able to activate transcription in a sequence-specific manner when heterodimerized with Max (Amati *et al.*, 1992; Kretzner *et al.*, 1992; Amin *et al.*, 1993).

The ability of Myc to function as a transcription factor prompted investigators to search for cellular targets of Myc activity. Several candidate Myc-induced genes have been identified using various methods (subtractive hybridization, differential display. The *ornithine decarboxylase* (*ODC*) gene possesses Myc E box sites and is activated by c-Myc. *ODC* is the rate-limiting enzyme in the synthesis of polyamines and is required for cellular progression through the cell cycle (Bello-Fernandez and Cleveland, 1992; Bello-Fernandez *et al.*, 1993; Pena *et al.*, 1993; Wagner *et al.*, 1993). Also, the cell cycle phosphatase *CDC 25A* is encoded by the *cdc 25* gene which is activated by c-Myc (Galaktionov *et al.*, 1996). Interestingly, the *lactate dehydrogenase-A* gene (*LDH-A*) is transcriptionally activated by c-Myc. The product of this gene is involved in normal anaerobic glycolysis and is found in increased levels in tumorigenic cells. Tumors with large mass have an internal environment deprived of oxygen, and thus overexpression of *LDH-A* by c-Myc could possibly atone for this deficiency (Shim *et al.*, 1997).

Besides Myc's ability to activate transcription from cellular promoters that contain Myc binding sites, Myc has also been shown to repress certain viral and cellular promoters. This repression by Myc corresponds to the presence of an initiator, or Inr, region (Smale and Baltimore, 1989) within the promoter of the Myc-downregulated gene. The adenovirus-2 major late promoter (Ziff and Evans, 1978) contains two E boxes recognized by Myc upstream of an Inr element. Interestingly, Myc exhibits a biphasic effect on this promoter: with increasing concentrations of Myc this promoter is initially activated, presumably through the Myc E box sequences. However, this activation is short-lived, as even higher concentrations of Myc result in inhibition of the promoter (Li *et al.*, 1994). To investigate this repression by Myc more closely, the Myc E box elements were removed from the adenovirus-2 major late promoter leaving only the Inr domain downstream of the TATA box. Increasing Myc expression inhibits transcription from this promoter in a dose-dependent manner, with no activation by Myc observed (Li *et al.*, 1994). Subsequently, other cellular promoters were identified as being targets of Myc-mediated repression, and most possess Inrs. First, the gene coding for the CCAAT/enhancer binding protein (C/EBP α), which is induced during differentiation of the adipogenic 3T3-L1 cell line, is downregulated when c-Myc is overexpressed. The *C/EBP α* gene contains an Inr element within its promoter region, and this interaction between Myc and the *C/EBP α* gene represents a mechanism by which Myc overexpression can contribute to inhibition of differentiation (Freytag and Geddes, 1992). Furthermore, Myc is able to directly downregulate the gene encoding thrombospondin-1. The implications of this inhibition on tumorigenesis are provocative: thrombospondin-1 inhibits angiogenesis due to its detrimental effect on migration, proliferation, and adhesion of the endothelial cells of blood vessels. Growing tumors rely on vascularization and a continuous blood supply, so suppression of thrombospondin-1 is advantageous to malignant cells (Tikhonenko *et al.*, 1996). Finally, Myc has recently been demonstrated to repress transcription of the *adrenomedullin* gene (Wang *et al.*, submitted). This gene codes for two peptides that function as vasodilators. Theoretically, this dilation could affect cell morphology, an aspect of cell physiology that is altered when a cell becomes transformed. Repression of *adrenomedullin* by Myc could contribute to the transformed phenotype typical of malignant cells. Inspection of the properties of these Myc-repressed genes indicates that suppression of growth-arrest genes and genes involved in inhibition of tumorigenesis are important negative targets of Myc action.

Another important function of Myc is induction of apoptosis under appropriate conditions. Evan *et al.* (1992) demonstrated in growing fibroblasts that, in combination with a block to cellular proliferation such as low growth factor conditions, overexpression of *c-myc* efficiently induces

programmed cell death, or apoptosis. In cells that have previously been growth arrested, expression of Myc is sufficient to cause cells to undergo programmed cell death. The paradoxical observation that Myc can induce a cell to both proliferate and self-destruct suggests the Myc protein is a key regulator of cell fate. Myc's role in inducing apoptosis is likened to a "fail-safe" mechanism to prevent a cell from becoming transformed. Myc production leading to tumorigenesis would only occur if cells already contained mutations that inhibit apoptosis or were located in an environment with adequate survival factors (reviewed in Packham and Cleveland, 1995). An interesting model has recently been proposed to explain Myc's role as both an inducer of cellular proliferation and apoptosis. As mentioned earlier, Myc activates expression of the *ornithine decarboxylase (ODC)* gene, and the protein product of this gene catalyzes the synthesis of polyamines (Bello-Fernandez *et al.*, 1993). This activity is required for cellular proliferation and therefore is a physiologically relevant target of Myc. However, high cellular levels of polyamines are also associated with cell death, due to the formation of reactive oxygen species, or ROS. Catabolism of polyamines generates potentially lethal amounts of ROS, mainly in the form of the superoxide ion, hydrogen peroxide, and the hydroxyl radical. Normally, detoxifying enzymes degrade the dangerous ROS before they can damage the cell; however, when high levels of ROS are generated, some cellular damage can occur, which is implicated in apoptosis (reviewed in Packham and Cleveland, 1995). c-Myc activation, which leads to increased *ODC* expression, may contribute to apoptosis via this mechanism (Packham and Cleveland, 1994). This supports other evidence generated that Myc-mediated apoptosis depends on formation of the Myc/Max heterodimer (Amati *et al.*, 1993). It is the Myc/Max complex that binds to the Myc E box within the *ODC* promoter to activate transcription of this gene.

Figure 1.1 depicts the structure of the v-Myc oncprotein and the regions essential to the various functions of Myc. As can be seen, many of these functions map to the amino terminus of the Myc protein. Upon closer examination of the amino terminus of Myc, two regions of complete sequence conservation are noted, Myc homology regions I and II (MHR I and MHR II; **Figure 1.2**). Conservation usually implies functional importance; indeed, the integrity of MHR I and MHR II is required for complete Myc function. For example, deletion of MHR II from c-Myc results in a protein that retains its ability to activate transcription from a reporter gene when fused to the GAL4 DB, indicating that residues comprising MHR II are dispensable for transcriptional activation. However, a full-length c-Myc protein in which precisely the same deletion has been generated is no longer able to cooperate with activated *ras* to transform cells (Brough *et al.*, 1995). MHR II, therefore, is essential to Myc's ability to induce cellular

transformation. Likewise, deletion of MHR II from full-length c-Myc abolishes Myc-mediated repression of the adenovirus-2 major late promoter (Li *et al.*, 1994). Finally, Myc-induced apoptosis also relies on MHR II integrity. Specifically, removal of residues comprising MHR II from wild-type c-Myc generates a protein unable to mediate apoptosis when the appropriate cellular conditions are met (Evan *et al.*, 1992). Results of these experiments indicate the importance of the integrity of the conserved domain MHR II to Myc function. A probable role for this conserved region in the functionally active Myc amino terminus is as an interaction interface for other cellular factors that mediate these functions of Myc.

Attempts by many laboratories to isolate Myc amino terminal-interacting factors have yielded several potential candidate proteins, the significance of which have not been fully determined. Initially, the retinoblastoma protein (Rb), a potent inhibitor of E2F-induced transactivation and well-characterized tumor suppressor (DeCaprio *et al.*, 1989), was shown to interact *in vitro* with the amino terminus of c-Myc (Rustgi *et al.*, 1991). Experiments to directly document the *in vivo* interaction between Rb and Myc have failed. However, the Rb-related protein p107 specifically interacts with the c-Myc amino terminus and results in the inhibition of c-Myc transcriptional activity (Gu *et al.*, 1994). Additionally, when cells are growth-arrested due to the action of p107, overexpression of c-Myc relieves this growth arrest, suggesting that Myc is targeted to achieve growth suppression by p107 (Beijersbergen *et al.*, 1994). Greater evidence supporting the functional interaction between c-Myc and p107 comes from the analysis of several mutant *c-myc* alleles derived from Burkitt's lymphomas. Proteins encoded by these *myc* genes contain many point mutations in the amino terminus of c-Myc. Interestingly, p107 still binds to these mutant Myc proteins but fails to suppress their transcriptional activities (Hoang *et al.*, 1995). These data support the role of p107 as a regulator of Myc activity. Next, the amino terminus of c-Myc binds specifically to TBP, the TATA-binding protein of the TFIID transcription initiation complex (Hateboer *et al.*, 1993; Maheswaran *et al.*, 1994). This is not surprising, since Myc has been shown to possess a TAD. Further analysis of this interaction yielded an interesting observation. When analyzed alone in solution, the c-Myc TAD exhibits no secondary structure. However, addition of TBP induced significant α -helical conformation in this region of c-Myc. This indicates that c-Myc undergoes a more structured configuration of its transactivation domain upon binding to a target factor, in this case TBP (McEwan *et al.*, 1996).

The functions of the Myc oncoprotein are varied and complex. While the elucidation of several candidate target genes of Myc action and potential interacting factors have provided a solid basis upon which to explain Myc function, these discoveries by no means fully elucidate the complete

function of the Myc protein *in vivo*. It has been the goal of the proceeding work to further analyze the highly transforming v-Myc protein to provide additional information regarding Myc's role as a central regulator of cellular activities.

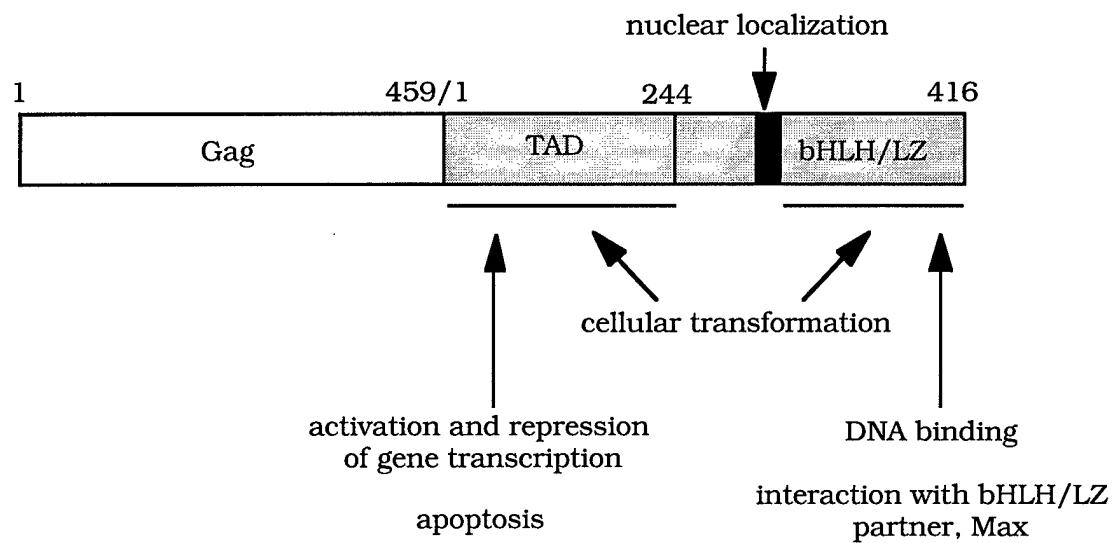


Figure 1.1 Diagram of the v-Myc oncprotein. Residues from the viral Gag protein comprise 459 amino acids of v-Myc, and these are represented by the white box. The transcription activation domain (TAD) and basic helix-loop-helix/leucine zipper (bHLH/LZ) regions of v-Myc are indicated as shaded boxes. The functions associated with the various portions of the v-Myc oncprotein are shown below and above the diagram.

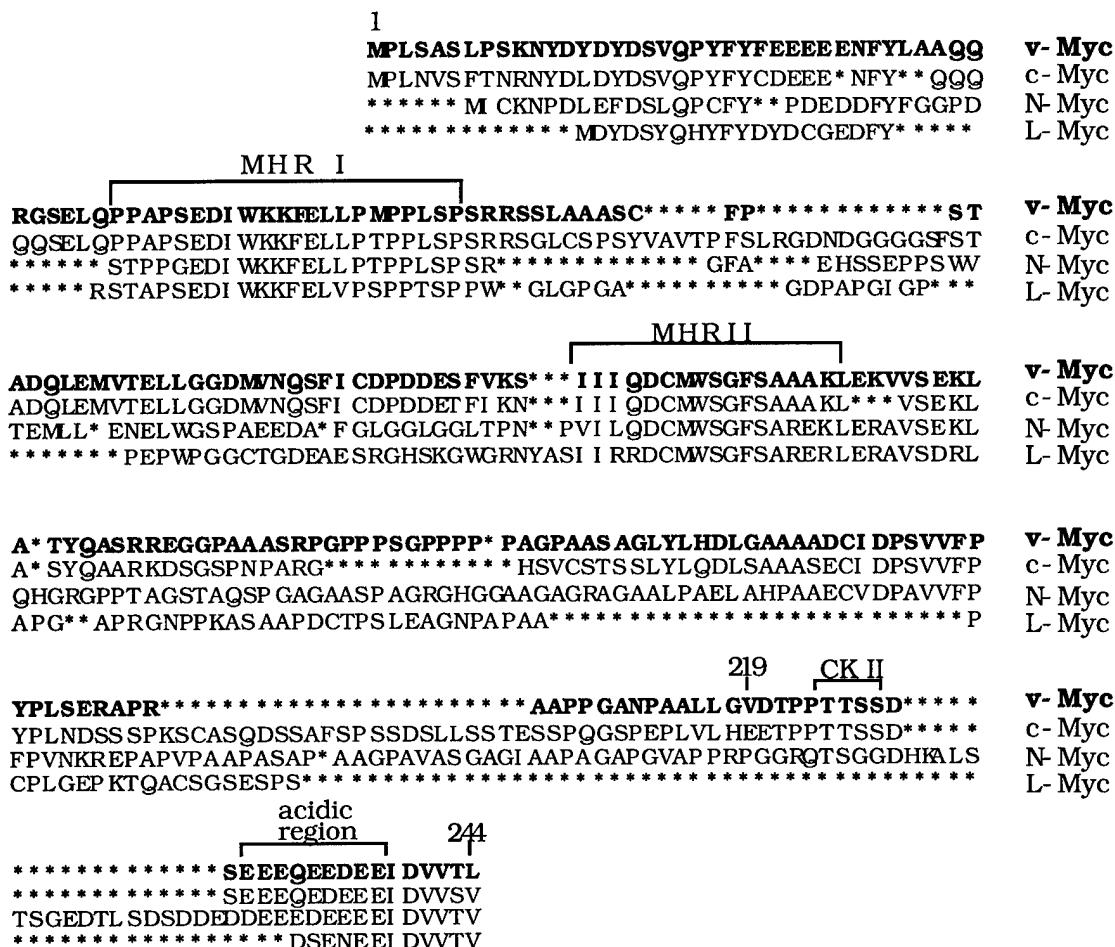


Figure 1.2 Transcription activation domains of the Myc family of oncoproteins. The “1” indicates the first residue of the v- and c-Myc protein. The highly conserved Myc homology regions I and II (MHR I and MHR II) are indicated. Residues 219 and 244 are designated, as these are important termination residues in the 5’ v-Myc deletions (Figure 3.2 and 3.5). This figure was adapted from Meichle *et al.* (1992).

SECTION 2: MATERIALS AND METHODS

Cell culture

C3H10T1/2 murine fibroblasts (ATCC # CCL226) and COS-1 African green monkey kidney cells (ATCC # CRL 1650) were maintained in basal modified Eagle medium (BME, GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (P/S). NIH 3T3 mouse embryo fibroblasts were maintained in Dulbecco's high glucose modified Eagle medium supplemented with 10% calf serum (CS), 100 U/ml penicillin, and 100 µg/ml streptomycin (P/S). Myc neo 13A cells (Davenport and Taparowsky, 1992), Ras neo 11A cells (Weyman *et al.*, 1988), and pDCR-B-ATF cells (Echlin *et al.*, in prep.) were maintained in BME plus 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (P/S), and 400 µg/ml geneticin (G418, GIBCO, Grand Island, NY).

Stable transfections

Stable transfection of C3H10T1/2 cells and NIH 3T3 cells was performed using the calcium phosphate/DNA precipitation method as previously described (Taparowsky *et al.*, 1987). 5 X 10⁵ cells were plated into 100 mm tissue culture dishes in complete medium (BME + 10% FBS + P/S for C3H10T1/2 cells or high glucose DMEM + 10% CS + P/S for NIH 3T3 cells) one day prior to transfection. Two hours before transfection, 100 µM chloroquine (Sigma, St. Louis, MO) was added to each dish of cells. Individual calcium phosphate/DNA precipitates containing appropriate amounts of DNA (see Figure Legends) were added to two 100 mm tissue culture dishes. Twenty-four hours post transfection, cells designated for cell transformation assays were split 1:3 and maintained in reduced-serum medium (5% FBS or CS), while C3H10T1/2 cells designated for colony formation assays were split 1:6 and maintained in BME plus 10% FBS, P/S, and 400 µg/ml G418. After 14 days of growth, stable cells were fixed with 5 ml of 100% methanol for 10 minutes and stained with 10 ml dilute (1:25) Giemsa (EM Diagnostic Systems, Darmstadt, Germany) for 15 minutes. Focus formation and/or colony formation was visually assessed. The efficiency of focus formation is expressed as a percentage, with either the amount of foci obtained from the co-expression of activated *H-ras* and *v-myc*, or *H-ras* alone (depending upon the experiment; see Figure Legends) set at 100%. For each cellular transformation assay, the efficiency of focus formation represents the average of at least three independent transfection experiments.

Generation of the stable cell line, OS-9-5

Myc neo 13A cells, into which the pMC29 *v-myc* gene and the neomycin-resistance gene have integrated (Davenport and Taparowsky, 1992), were stably transfected with 100 ng of the plasmid supplying resistance to the antibiotic puromycin (pBABE puro; gift of C. Marshall, Chester Beatty Labs, London, England) and 1 μ g of OS-9(1-667). Twenty-four hours post-transfection, the cells were split 1:6 and maintained in BME supplemented with 10% FBS, 400 μ g/ml G418, 5 μ M puromycin, 100 U/ml penicillin, and 100 μ g/ml streptomycin. After 10 days, when distinct colonies had formed, individual colonies were picked by isolation via a cloning ring, trypsinization, and transfer to a 35 mm tissue culture dish in the above-described medium. Colonies were grown and expanded. Detection of OS-9 expression (via the HA epitope tag) was assessed by Western blotting (described below), and clones that had detectable OS-9 expression levels were analyzed further.

Transient transfections and chloramphenicol acetyl transferase (CAT) assays

Transient transfections were performed using the calcium phosphate/DNA precipitation method as described previously (Min and Taparowsky, 1992). For the GAL4-v-Myc activation studies, 5 μ g of GAL4 fusion protein, 5 μ g of (GAL4)₅E1B TATA CAT reporter, and 5 μ g of the β -galactosidase expression vector, RSV-LacZ (Jaynes *et al.*, 1986) were added to individual 100 mm tissue culture plates containing 5 X 10⁵ cells seeded the previous day. For the experiments utilizing the (MBS)₃ TK CAT reporter plasmid, 5 μ g of reporter and 12.5 μ g of pMC29 *v-myc*, pMC29(Δ 220-245), or VP16-v-Myc was added to individual plates. For the studies using the AM-CAT reporter, 2.5 μ g AM-CAT, 5 μ g pMC29 v-Myc, and 10 μ g OS-9(1-667) were used to make each calcium phosphate/DNA precipitate. Finally, when the p(LexA)₈(GAL4)₅CAT reporter was used, 5 μ g reporter, 1 μ g LexA activator, and 5 μ g of each GAL4 fusion were used. Five hours following the addition of precipitates, cells were osmotically shocked with 20% glycerol (in serum-free BME) for 2 minutes, rinsed with serum-free BME, and refed complete medium. Cells then were maintained in complete medium for an additional 48 hours, at which time they were rinsed in 5 ml cold CMF saline, pH 7.4 (130 mM NaCl, 1.5 mM KH₂PO₄, 8.0 mM Na₂HPO₄, 2.7 mM KCl) and harvested by scraping in 1 ml cold CMF saline. Cells were pelleted, washed with 1 ml cold 0.25 M Tris, pH 8.0 and resuspended in 230 μ l of Tris/EDTA buffer (0.25M Tris, 5 mM EDTA, pH 8.0) at 4°C. Cell membranes were disrupted by sonication for 20 seconds, and cell extracts were cleared of cellular debris by centrifugation at 4°C for 15 minutes. The β -galactosidase activity measured in each sample was used to normalize the amount of extract necessary to assay the CAT activity of each extract as described (Yutzey *et al.*, 1989). Cell extracts not containing the RSV-LacZ plasmid were prepared as outlined above

except that each extract was normalized to total protein content (Bio-Rad Protein Assay) prior to the CAT assay. Percent conversion of ¹⁴C-chloramphenicol (ICN) to the acetylated form by the CAT enzyme was determined by liquid scintillation counting. All assays were maintained within the linear range of CAT activity.

Transient Transfection and Luciferase Assays

C3H10T1/2 cells were seeded in 6-well dishes at a cellular density of 6×10^4 cells per well 24 hours prior to transfection. Individual calcium phosphate/DNA precipitates containing 5 μ g pMLb-luc reporter, 2, 5, or 10 μ g of pMC29 *v-myc* or *USF* (Li *et al.*, 1994), and 5 μ g OS-9(1-667) were added to each well. Five hours after addition of the precipitates, each well was drained, rinsed twice with cold CMF saline, and refed 2 ml complete media (BME + 10%FBS + P/S). After 48 hours, cells were rinsed with 2 ml cold PBS, pH7.4 (2.7 mM KCl, 1.5 mM KH₂PO₄, 136.9 mM NaCl, 15.2 mM Na₂HPO₄) and harvested by addition of 250 μ l lysis buffer (25 mM Tris pH7.8, 8 mM EDTA, 10% glycerol, 1% Triton X-100) to each well for 15 minutes. Cell extracts were analyzed with the luciferase assay kit (Promega) in a luminometer (Lumat # LB 9501) followed by normalization of total protein content (Bio-Rad Protein Assay).

Transient transfections and Western blot analysis

COS-1 cells, seeded one day prior to transfection at 1×10^6 cells per 100 mm dish, were transiently transfected with 10 μ g of the intended GAL4-*v-Myc*, pMC29, or OS-9 plasmid (see Figure Legends). Two days after transfection, COS-1 cells were harvested in 25 μ l 4X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (200 mM Tris pH6.8, 400 mM dithiothreitol, 8% SDS, 0.4% bromophenol blue, 40% glycerol). Equal amounts of whole cell extract were separated by 10% SDS-PAGE and transferred to nitrocellulose filter paper as described (Ramocki *et al.*, 1997). Nonspecific protein binding was blocked by incubation in 5% nonfat dry milk in TBST (10 mM Tris pH8.0, 150 mM NaCl, 0.1% Tween 20) followed by incubation in 5% nonfat dry milk in TBST containing a 1:200 dilution of anti-GAL4 antibody (RK5C1; Santa Cruz), a 1:5000 dilution of anti-Gag antibody (3C2; gift of S. Konieczny, Department of Biological Sciences, Purdue University, West Lafayette, IN) or a 1:5000 dilution of anti-HA antibody (3F10; Boehringer Mannheim). After multiple washes in TBST, protein expression was detected by using a peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL kit; Amersham).

Growth curve assays

For the determination of cellular growth rates of the various cell lines used in these studies, multiple 60 mm dishes containing growth medium (BME + 10% FBS +P/S) were seeded with 1 X 10⁴ cells on Day 0 as previously described (Davenport and Taparowsky, 1992). On Day 1 and every three days thereafter, all cells were fed growth medium. Duplicate dishes of each cell line were counted daily on Days 2 through 10 by trypsinization and use of a hemacytometer. The average number of cells per 60 mm dish was plotted versus the time course of the experiment. Each plot of the rate of cellular growth for the cells examined (C3H10T1/2, Ras neo 11A, Myc neo 13A, OS-9-5, and pDCR-B-ATF) is the result of three independent counting experiments.

Nuclear extract isolation

Nuclear extracts were isolated from transiently transfected COS-1 cells as described (Lassar *et al.*, 1991). Forty-eight hours post transfection cells were rinsed three times with CMF saline at 4°C and harvested with 2 ml/plate lysis buffer (20 mM Hepes pH7.6, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 100 µg/ml aprotinin). The harvested nuclei were resuspended in 50 µl nuclear extraction buffer (20 mM Hepes pH7.6, 20% glycerol, 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 100 µg/ml aprotinin). The protein concentration of each extract was determined using the Bio-Rad Protein Assay.

Electrophoretic mobility shift assays (EMSA)

The probe used for EMSA consisted of the HindIII/XbaI fragment from (GAL4)₅E1B TATA CAT (Lillie and Green, 1989) which excises the five tandem GAL4 protein binding sites. The GAL4 probe was isolated, purified, and quantified by separation on a 1% agarose gel. The probe was end-labeled using Klenow DNA polymerase and [α -³²P]-dCTP (6000 Ci/mmol; Amersham) in the presence of unlabeled dATP, dGTP, and dTTP. All probes were purified as described previously (Sambrook *et al.*, 1989) and approximately 2 x 10⁴ cpm of each probe were incubated with nuclear extracts at 4°C for 30 minutes. The binding buffer for the nuclear extracts consisted of 25 mM Hepes (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 5 mM DTT, 5 mM MgCl₂, 10% glycerol and 2 µg poly [dI-dC]. The DNA binding profiles were resolved by electrophoresis through a 5% non-denaturing polyacrylamide gel in medium high ionic strength (MHIS) buffer (12.5 mM Tris pH 8.5, 95 mM glycine, 0.5 mM EDTA). Electrophoresis was conducted for 3 hours at 100V after which time the gel was vacuum dried and exposed to X-ray film overnight with an intensifying screen at -80°C.

Metabolic labeling of COS-1 cells

Transiently transfected COS-1 cells were grown for forty-eight hours post transfection (see above) at which time cells were rinsed with 5 ml cold CMF saline and fed 10 ml low glucose DMEM plus 1% FBS, 1% L-glutamine, 1% sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin minus methionine [Met (-) medium] for one hour. After this period of starvation, cells were incubated in 3 ml Met (-) medium plus 0.4 mCi per plate Trans-³⁵S Label (ICN) in a sealed modular incubator chamber at 37°C for 4 hours. After the labeling period, plates were removed from the incubation chamber in a fume hood and rinsed twice with cold CMF saline. 200 µl of RIPA buffer (10 mM Tris pH7.5, 150 mM NaCl, 2 mM EDTA, 0.1% DOC, 0.5% NP-40, 0.05% SDS 1 mM PMSF, and 30 µl/ml aprotinin) was added to each plate, and the cells were collected. Passage through an 18 gauge needle and 3 ml syringe three times efficiently homogenized the suspension.

Co-immunoprecipitation procedures

Co-immunoprecipitation procedure I utilizes the labeled cell extracts isolated from transiently transfected COS-1 cells outlined in the above section. Each sample was divided into two 1.5 ml screw-cap Eppendorf tubes, and 10 ml of anti-Gag antibody (3C2; gift of S. Konieczny) and 25 µl anti-HA antibody (3F10; Boehringer Mannheim) was added to each individual tube. Samples were incubated end-over-end for 1.5 hours at 4°C. 100 µl of RIPA buffer-equilibrated, 50% slurry, Affi Gel Protein A agarose beads (Bio-Rad) were added to each sample and rotated end-over-end for an additional one hour at 4°C. Samples were rinsed two times with 1 ml RIPA buffer, and 10 µl of 4 X SDS-PAGE sample buffer was added to each tube. After heating sample at 95°C for 10 minutes to remove protein complexes from the beads, proteins were separated by 10% SDS-PAGE, and the gel was vacuum-dried and exposed to X-ray film overnight. Co-immunoprecipitation procedure II involves utilizing non-labeled COS-1 cellular extracts.

Transiently transfected COS-1 cells were rinsed twice in 5 ml cold CMF saline and harvested in 300 µl RIPA buffer (10 mM Tris, pH7.5, 150 mM NaCl, 2 mM EDTA, 0.1% DOC, 0.5% NP-40, and 0.04% SDS) per plate. Two plates per experimental group were harvested together, and samples were passed through an 18 gauge needle and 3 ml syringe five times to homogenize the suspension. Another 300 µl RIPA was added to each tube along with 25 µl anti-HA antibody (3F10). Samples were rotated end-over-end for two hours at 4°C. 100 µl of RIPA buffer-equilibrated, 50% slurry, Affi Gel Protein A agarose beads were added to each sample and rotated end-over-end for an additional three hours. Samples were rinsed twice with 1 ml of RIPA buffer, and 5 µl of 4 X SDS-PAGE sample buffer plus 15 µl of H₂O were added to each

tube. Samples were heated for 10 minutes at 95°C, separated by 10% SDS-PAGE, and transferred to nitrocellulose filter paper. The non-specific binding of proteins was blocked by incubation in 5% nonfat dry milk in TBST for one hour. Protein expression was then analyzed by incubation of the membrane in a 1:5000 dilution of anti-Gag antibody (3C2) in 5% milk followed by several washes in TBST, incubation with a peroxidase-conjugated secondary antibody and finally, detection by enhanced chemiluminescence (ECL kit, Amersham).

FACS analysis of cell lines

The cell lines analyzed for apoptosis were the parental fibroblasts C3H10T1/2, the v-Myc-overexpressing cell line Myc neo 13A (Davenport and Taparowsky, 1992), and the v-Myc/OS-9(1-667)-overexpressing cell line OS-9-5 (see ***Generation of the stable cell line, OS-9-5*** in Materials and Methods). One plate of each cell line was allowed to grow to near confluence in growth medium (BME + 10%FBS + P/S); another plate of each cell type was allowed to grow to near confluence and then maintained in starvation medium (BME + 0.5% FBS + P/S) for 48 hours. At this time, cells that had detached from the plates were collected, and the adherent cells of each type in each condition were collected by trypsinization. The suspended and adherent cells were combined and analyzed together. Cells were fixed by resuspension in 1 ml of 1% formaldehyde in PBS (2.7 mM KCl, 1.5 mM KH₂PO₄, 136.9 mM NaCl, 15.2 mM Na₂HPO₄), followed by incubation at 4°C for 15 minutes, rinsing with PBS, and resuspension in 500 µl of 70% ethanol. Cells were kept in 70% ethanol at 4°C overnight. After removal of ethanol, cells were resuspended in PBS and counted. To perform the *in situ* Terminal Deoxynucleotidyl Transferase (TDT) Assay, 1 X 10⁶ cells were aliquotted into Eppendorf tubes and centrifuged for 15 minutes, drained, and resuspended in 50 µl of cacodylate buffer (0.2 M potassium cacodylate, 25 mM Tris, pH 6.6, 0.25 mg/ml bovine serum albumin, 2.4 mM cobalt chloride, 100 U/ml terminal deoxynucleotidyl transferase, and 0.5 nM biotin-16 dUTP [Boehringer Mannheim]). Cells were incubated at 37°C for 30 minutes, drained, rinsed in PBS, drained, and resuspended in 100 µl of saline-sodium citrate buffer (4X SSC buffer [0.6 M NaCl, 0.06 M NaCl]) containing 2.5 µg/ml of fluoresceinated avidin, 0.1% Triton X-100, and 5% (w/v) non-fat dry milk. To stain the cells and measure fluorescence, cells were drained, rinsed in 0.1% Triton X-100 in PBS, drained again, and resuspended in 1 ml of PI buffer (5 µg/ml Propidium Iodide [Sigma], 0.1% RNase A [Sigma] in PBS). Fluorescence was measured at 525 nm (green) to detect labeled DNA strand breaks and at 620 nm (red) to determine the cell cycle position of the samples.

SECTION 3: ANALYSIS OF THE v-MYC TAD

Introduction

The Myc protein has many features of a typical eukaryotic transcription factor. Specifically, it is a nuclear protein (Stone *et al.*, 1987; Dang *et al.*, 1989) that contains both a DNA binding, basic domain and a helix-loop-helix/leucine zipper region that mediates protein-protein interactions (reviewed in Meichle *et al.*, 1992) in the carboxyl terminus of the protein. Additionally, the amino termini of Myc proteins have regions of high proline content and stretches of glutamine and acidic residues that are characteristic of many known regulators of transcription (reviewed in Ptashne, 1988; Mitchell and Tjian, 1989). The demonstration that residues in both the amino terminus and carboxy-terminal basic helix-loop-helix/leucine zipper of Myc are necessary for cellular transformation (Stone *et al.*, 1987) prompted several groups to examine the amino terminus of the c-Myc protein and to determine if these sequences could function as a transcription activation domain.

Kato *et al.* (1990) initially began work on the c-Myc amino terminus. By fusing the first 262 residues of c-Myc to the DNA binding domain of the yeast protein GAL4, they were able to demonstrate that these Myc residues activated transcription of a reporter plasmid containing GAL4 protein binding sites. Importantly, these same residues of c-Myc are responsible for Myc's ability to induce cellular transformation (Stone *et al.*, 1987). Further studies showed that certain subregions within the amino terminus of c-Myc were even stronger activators of transcription than the full-length c-Myc amino terminus (Kato *et al.*, 1990). This was the first evidence that the Myc amino terminus was capable of functioning as a transcription activation domain.

Work by a former graduate student in the lab, Dr. Soyoung Min, identified a similar transcription activation domain within the amino terminus of the highly transforming v-Myc oncoprotein. Specifically, the first 244 residues of v-Myc activate transcription when fused to the GAL4 DNA binding domain (Min and Taparowsky, 1992). Like c-Myc, the v-Myc amino terminus can also be subdivided into regions that have stronger activation potential than the full-length v-Myc TAD. The TAD of v-Myc also is absolutely required for v-Myc's ability to cooperate with H-Ras to transform cells (Min and Taparowsky, 1992). Since this region is vital to Myc function, a more thorough analysis of the Myc TAD was undertaken to provide a detailed map of the v-Myc transcription activation domain.

Results

The extreme amino terminus of the v-Myc TAD is a potent transactivator

To examine the transcriptional properties of subregions of the v-Myc transcription activation domain (TAD), deletion mutagenesis of the amino terminus, which encodes the v-Myc TAD, was performed as outlined in Materials and Methods. A series of 3' and 5' deletions, diagrammed in **Figures 3.1** and **Figure 3.2**, were generated and used to assess the transcriptional activity of various domains of the v-Myc TAD. C3H10T1/2 murine fibroblasts were transiently transfected with 5 mg of each GAL4-v-Myc fusion, and the resulting CAT activity of harvested cell extracts was determined. As can be seen in **Figure 3.3**, the first 27 amino acids of v-Myc (-32-27) are sufficient to recapitulate the activity of the full-length v-Myc TAD (-32-219).

Interestingly, GAL4-v-Myc mutants (-32-97) and (-32-112) are extremely strong activators of transcription, nearly two-fold more active than the full-length TAD (**Figure 3.3**). To begin with, this suggests that the conformation of the residual v-Myc protein sequences in these deletions is not adversely affected to render them ineffective transcriptional activators. Next, examination of the remaining 3' deletions of the v-Myc TAD reveals a decrease in transcriptional activity as increasingly larger portions of the activation domain are assayed. For example, v-Myc residues -32-134 are approximately 50% as effective as the full-length v-Myc TAD and nearly three-fold less active than v-Myc residues -32-112. This could suggest that an inhibitory region exists beyond amino acid 112 of v-Myc. The most notable difference between the v-Myc deletions (-32-112) and (-32-134) is the inclusion of MHR II in the latter protein (**Figure 3.1**). In fact, every deletion analyzed that does contain MHR II is less potent an activator than the full-length v-Myc TAD (**Figure 3.3**). Alternatively, perhaps inclusion of sufficiently longer pieces of the v-Myc TAD (deletions -32-134, -32-139, -32-142, -32-148, -32-162, -32-179, and -32-182) results in proteins with altered structure when compared to the full-length v-Myc TAD. This alteration could affect the ability of these mutants to properly activate transcription of the reporter gene utilized.

v-Myc residues 219-244 impart inhibitory effects on v-Myc transcriptional activation

Besides the 3' deletions of the v-Myc TAD analyzed in **Figure 3.3**, 5' deletions were also generated by the same protocol and analyzed in C3H10T1/2 cells. **Figure 3.2** depicts the v-Myc residues remaining after the deletion procedure that are fused downstream of the DNA binding domain of the yeast GAL4 protein. Each 5' v-Myc mutant extends to amino acid 244. The ability of these deletions to activate transcription from the (GAL4)₅ E1B TATA CAT reporter was assayed and is presented in **Figure 3.4**. As can be seen, none of the v-Myc TAD mutants tested were even 50% as active as the wild-type v-Myc activation domain. Because such low

activity was obtained from all of the GAL4-5'-v-Myc deletions, these proteins were further modified to terminate at v-Myc residue 219 rather than residue 244 (**Figure 3.5**). As outlined in the Materials and Methods, a SalI restriction site at v-Myc residue 219 facilitated the removal of the terminal 25 amino acids of each of these GAL4-v-Myc fusions. The modified 5' v-Myc mutants were then similarly tested for their ability to activate transcription from a reporter gene. **Figure 3.6** demonstrates the transcriptional activities of these mutants. When compared to the activities of their longer counterparts in **Figure 3.4**, each modified deletion (except 107-219) is at least twice as effective at activating transcription of the reporter than the original protein. As previously mentioned, v-Myc residues 107-219 were not able to transactivate the reporter. This suggests that residues in the amino terminus of the v-Myc TAD are necessary for the ability of the protein to be transcriptionally active. These results also imply that v-Myc residues 219-244 impart an inhibitory function on Myc's ability to activate transcription. In order to assure that protein stability could not account for the differences seen between different v-Myc deletions, the levels of protein expression via Western blot detection were analyzed. **Figure 3.7** depicts the *in vivo* expression of selected GAL4-v-Myc deletions. Whole cell extracts of transiently transfected COS-1 cells were harvested and incubated with an anti-GAL4 antibody as described in Materials and Methods. All proteins are equivalently expressed, and even the two forms of the 5' v-Myc deletions are expressed well in cells. This demonstrates that protein instability due to either the deletion protocol or the addition of the GAL4 DNA binding domain does not occur. To further these studies, EMSA was performed on selected GAL4-v-Myc deletions (**Figures 3.8** and **3.9**). A probe consisting of five GAL4 protein binding sites was labeled with ³²P and incubated with nuclear extracts harvested from COS-1 cells transiently transfected with 10 mg of the selected plasmids. Samples were then resolved on a 5% non-denaturing polyacrylamide gel as described in Materials and Methods. **Figure 3.9** shows that both GAL4-v-Myc(12-244) and GAL4-v-Myc(12-219) bind DNA equally well. This indicates that removal of v-Myc residues 220-244 does not affect the ability of the GAL-v-Myc fusion proteins to bind DNA. Taken in conjunction with the Western blot experiments (**Figure 3.7**), this implies v-Myc residues 220-244 are inhibitory to Myc's transactivation potential and do not modify v-Myc protein stability or DNA binding.

Deletion of residues 220-245 from full-length v-Myc increases Myc transactivation potential
In order to examine the role of amino acids 219-244 in the context of the full-length v-Myc protein, these residues (220-245) were removed from v-Myc as detailed in Materials and Methods. Removal of these 25 amino acids from the protein does not affect protein stability. Western blot analysis of transiently transfected COS-1 cells illustrates via incubation with an

anti-Gag antibody that both v-Myc proteins are expressed well *in vivo* (Figure 3.10). The ability of each of these proteins to activate a Myc-responsive reporter is shown in Figure 3.11. The Myc-responsive reporter (MBS)₃ TK CAT, in which three tandem copies of the Myc binding site (or Myc E box) is located upstream of the minimal thymidine kinase promoter and CAT gene, was utilized to detect transcriptional activity of these Myc proteins that contain their natural DNA binding and oligomerization domains. The reporter exhibits moderately high basal levels alone, presumably due to endogenous c-Myc protein. Therefore, addition of v-Myc only slightly activates this reporter. Figure 3.11 indicates that addition of v-Myc increases the activity of this reporter approximately 1.5-fold. However, the v-Myc protein in which residues 220-245 have been deleted activates transcription nearly 3-fold. This correlates with the results obtained using GAL4-5' v-Myc deletions. To examine if v-Myc residues 220-245 affect other functions of the Myc protein, Myc's ability to repress transcription from certain responsive reporters was analyzed. The AM-CAT reporter contains 2 kb of 5' promoter region of the *adrenomedullin* gene, identified as candidate gene for Myc-mediated repression, located upstream of the CAT gene (see Materials and Methods). Figure 3.12 illustrates the high level of CAT activity exhibited by this reporter alone, while co-expression of v-Myc reduces this activation approximately 80%. v-Myc(D220-245) represses the activity of AM-CAT to the same extent, implying that v-Myc residues 220-245 do not affect Myc's ability to repress transcription. As a final means to analyze the impact of deletion of amino acids 220-245 from the v-Myc protein, the ability of this v-Myc mutant to cooperate with an activated H-Ras oncoprotein and induce cellular transformation was studied. As described in Materials and Methods, C3H10T1/2 cells are stably transfected with activated H-ras, and as shown in Figure 3.13, a low level of focus formation occurs. However, co-expression of H-ras and *v-myc* results in an increase in cellular transformation, and this value is set at 100%. Co-expression of H-ras and *v-myc(D220-245)* results in a slight increase in focus formation over the level of transformation obtained with H-ras and *v-myc* (130% and 100%, respectively). This suggests that in addition to affecting Myc's transcriptional properties, residues 220-245 also impact Myc's ability to transform cells.

The functions of the v-Myc TAD cannot be completely recapitulated by replacement with a heterologous TAD

To examine if merely the activation of Myc-responsive genes is sufficient to induce cellular transformation, a chimeric protein was engineered in which the strong activation domain (residues 411-415) of the viral activator VP16 were fused in-frame to the basic helix-loop-helix/leucine zipper (bHLH/LZ) region of v-Myc (residues 244-416; see Materials and Methods). This chimeric protein contains the natural DNA binding and heterodimerization domains of v-

Myc; therefore, it should be able to complex with Myc's binding partner Max and properly interact with Myc E box-containing DNA. The function of the v-Myc TAD can then be assessed. **Figure 3.14** demonstrates that the VP16-v-Myc fusion protein can activate transcription from a reporter containing Myc binding sites. In these experiments, wild-type v-Myc activates the (MBS)₃ TK CAT reporter approximately 3-fold, and the VP16-v-Myc fusion protein activates the reporter nearly 2.5-fold. This significant, reproducible activation of the reporter suggests that the VP16-v-Myc chimera can interact with Max and properly bind to Myc E box DNA, and it also suggests that the TAD of VP16 functions to activate transcription when fused to the Myc bHLH/LZ. However, despite its ability to activate transcription of Myc-responsive reporters (and presumably genes), this VP16-v-Myc fusion protein cannot cooperate with H-Ras to transform cells. **Figure 3.15** indicates the results of stable transfection of C3H10T1/2 cells. Neither wild-type v-Myc or VP16-v-Myc alone can transform cells, but expression of activated H-Ras induces a low level of focus formation. This level is increased by 2.5-fold when H-Ras and v-Myc are co-expressed in cells; however, co-expression of H-Ras and VP16-v-Myc does not augment H-Ras-mediated cellular transformation (**Figure 3.15**).

Discussion

The transcription activation domain of the Myc family of proteins has been analyzed for its ability to contribute to Myc's varied functions. The v-Myc TAD has been shown to consist of two large subregions (-32-42 and 90-219), each of which is a more potent activator of transcription when fused to GAL4 DNA binding domain than the full-length v-Myc activation domain alone (Min and Taparowsky, 1992). The amino terminus of v-Myc is also essential for Myc's other cellular functions including transformation (Stone *et al.*, 1987), repression of certain viral and cellular promoters (Li *et al.*, 1994;), and apoptosis (Evan *et al.*, 1992). The existence of two highly conserved domains throughout all Myc family members (reviewed in Henriksson and Luscher, 1996 and Marcu *et al.*, 1992) also suggests that the transcription activation domain is an essential component of Myc activity. A detailed analysis of the highly transforming v-Myc TAD should therefore help to reveal more information concerning the role of the Myc activation domain in all of these important Myc-mediated activities.

Deletion mutagenesis of the v-Myc TAD reveals that the extreme amino terminus of v-Myc is a stronger activator of transcription than subsequently longer portions of the v-Myc TAD (**Figure 3.3**). While this could be the result of structural changes as a consequence of the mutations, this

could also indicate that MHR II is important in modulating the transcription activation properties of the v-Myc TAD. Indeed, experiments outlined in Chapter 5 of this manuscript describe OS-9, a MHR II-interacting protein, and its repressive effect on Myc-mediated transcriptional activation. Significantly, deletion of MHR II from the c-Myc TAD does not inhibit its ability to activate transcription; on the contrary, this c-Myc deletion is greater than two-fold more transcriptionally active than the full-length c-Myc TAD (Brough *et al.*, 1995). This is additional evidence that MHR II functions to negatively impact Myc transcriptional activation.

Further analysis of v-Myc amino terminal mutants reveals that removal of residues 220-245 from the TAD significantly increases transcriptional activity by both GAL4-v-Myc fusion proteins (**Figures 3.4 and 3.6**) and in the full-length v-Myc protein (**Figure 3.11**). While these residues do not affect Myc-mediated repression, a slight but reproducible increase in cellular transformation of murine fibroblasts is observed when these residues are deleted from v-Myc (**Figure 3.13**). As can be seen in **Figure 1.2**, two areas of significance occur between v-Myc residues 220-245. The first is an *in vivo* casein kinase II (CK II) phosphorylation site, and the other is an extremely acidic stretch of amino acids. Mutation of the CK II phosphorylation site does not affect c-Myc function (S. Hann, personal communication). While many transcription factors, including Myc, are characterized by stretches of acidic, negatively charged amino acids (Mitchell and Tjian, 1989), this is also a motif present in some repressors of transcription. For example, a very powerful transcriptional repression domain called the *Kruppel*-associated box (KRAB) is a highly acidic motif found in the amino terminus of approximately one-third of all mammalian *Kruppel*-related zinc finger proteins (Hanna-Rose and Hansen, 1996). The effect of deletion of residues 220-245 of v-Myc parallels another study in which a similar mutation in v-Myc (D220-239) was analyzed for its ability to transform avian cells. While this deletion was still effective at transforming chicken embryo cells, it was no longer able to transform chicken bone marrow cells (Heaney *et al.*, 1986). Also, analysis of a v-Myc mutant in which a larger portion of the v-Myc TAD had been deleted (residues 138-245) resulted in a v-Myc protein exhibiting a “delayed” transforming phenotype. This v-Myc protein retained its ability to transform chicken embryo cells; however, formation of foci appears 2 to 3 days after wild-type v-Myc-induced foci appeared (Farina *et al.*, 1992). This mutant transformed chicken embryo macrophages much more efficiently than chicken embryo fibroblasts. Experiments such as these indicate the amino terminus of v-Myc specifies certain regulatory interactions and further implicates the importance of amino acids 220-245 to v-Myc function. Significantly, no such experimentation has been performed on c-Myc.

Finally, the functions of a transcription activation domain in general, and the v-Myc TAD specifically, were analyzed via a chimeric protein that retained the v-Myc DNA binding and oligomerization domains yet was substituted for the v-Myc TAD by that of a heterologous activation domain. **Figure 3.14** demonstrates that this fusion protein between the VP16 TAD and the carboxy terminus of v-Myc still activates transcription from a Myc-responsive reporter (and therefore still interacts with the Myc binding partner Max and recognizes Myc E box DNA), it is no longer effective at cooperating with activated H-Ras to transform fibroblasts (**Figure 3.15**). The protein expression level of the VP16-v-Myc fusion was not analyzed in these experiments, however. Presumably, VP16-v-Myc could be an even stronger activator of transcription than detected but highly degraded in cells. (These results were also confirmed and published by Brough *et al.*, 1995. In this study, VP16-v-Myc was found to be equivalently expressed as wild-type v-Myc.) This result implies that the v-Myc transcription activation domain encodes a unique function besides transcriptional activation that accounts for its ability to transform cells. The most likely explanation of this function is protein-protein interactions that are special to the v-Myc TAD that cannot be replaced by another functional, potent TAD.

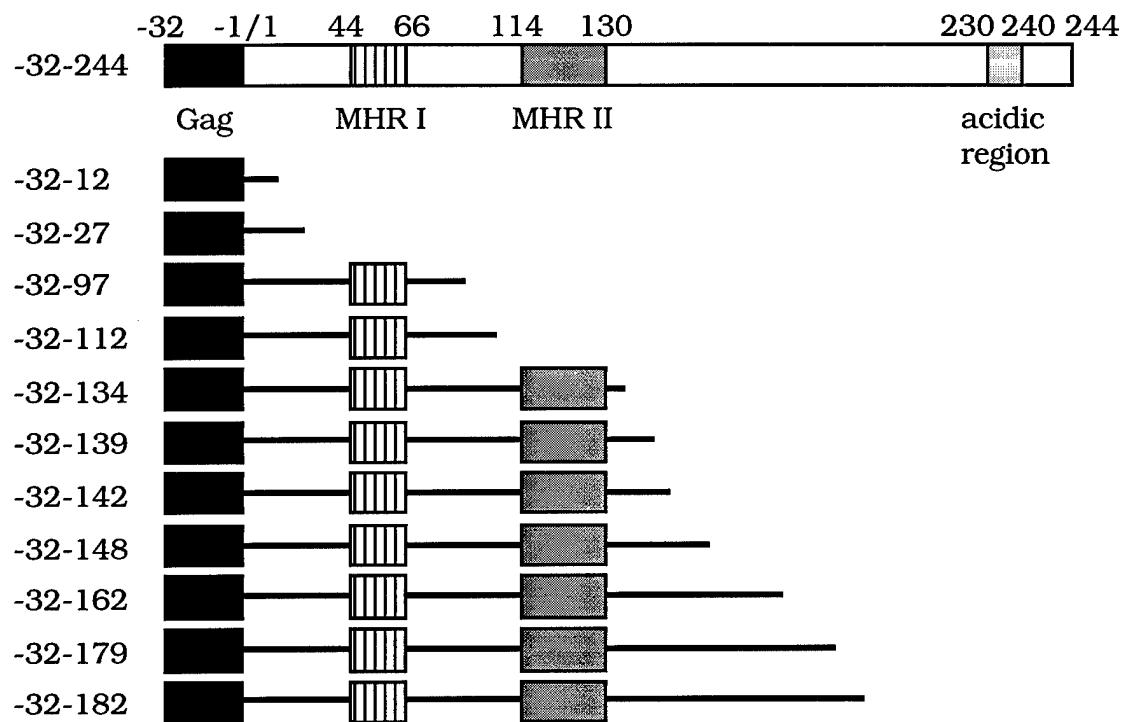


Figure 3.1 Schematic diagram of nested 3' deletions of the v-Myc TAD. Pictured are the protein sequences remaining in each construct. Each v-Myc protein is fused at the amino terminal Gag sequence to the GAL4 DNA binding domain. Details on the construction of these v-Myc TAD deletions can be found in the Materials and Methods.

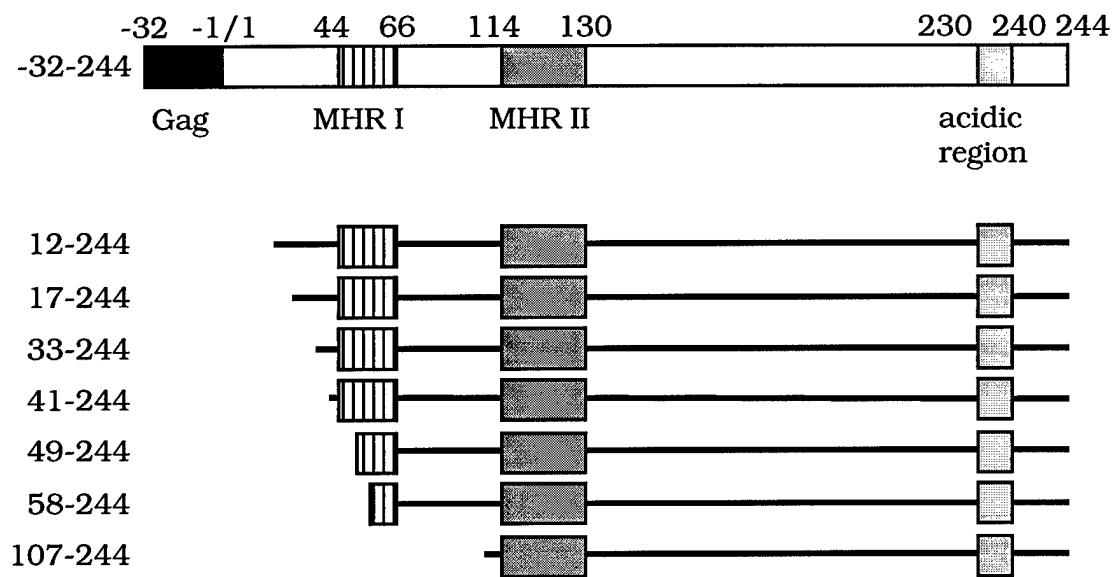


Figure 3.2 Schematic diagram of nested 5' deletions of the v-Myc TAD. Pictured are the protein sequences remaining in each construct. Each v-Myc protein is fused at the amino terminus to the GAL4 DNA binding domain. Details on the construction of these v-Myc TAD deletions can be found in the Materials and Methods.

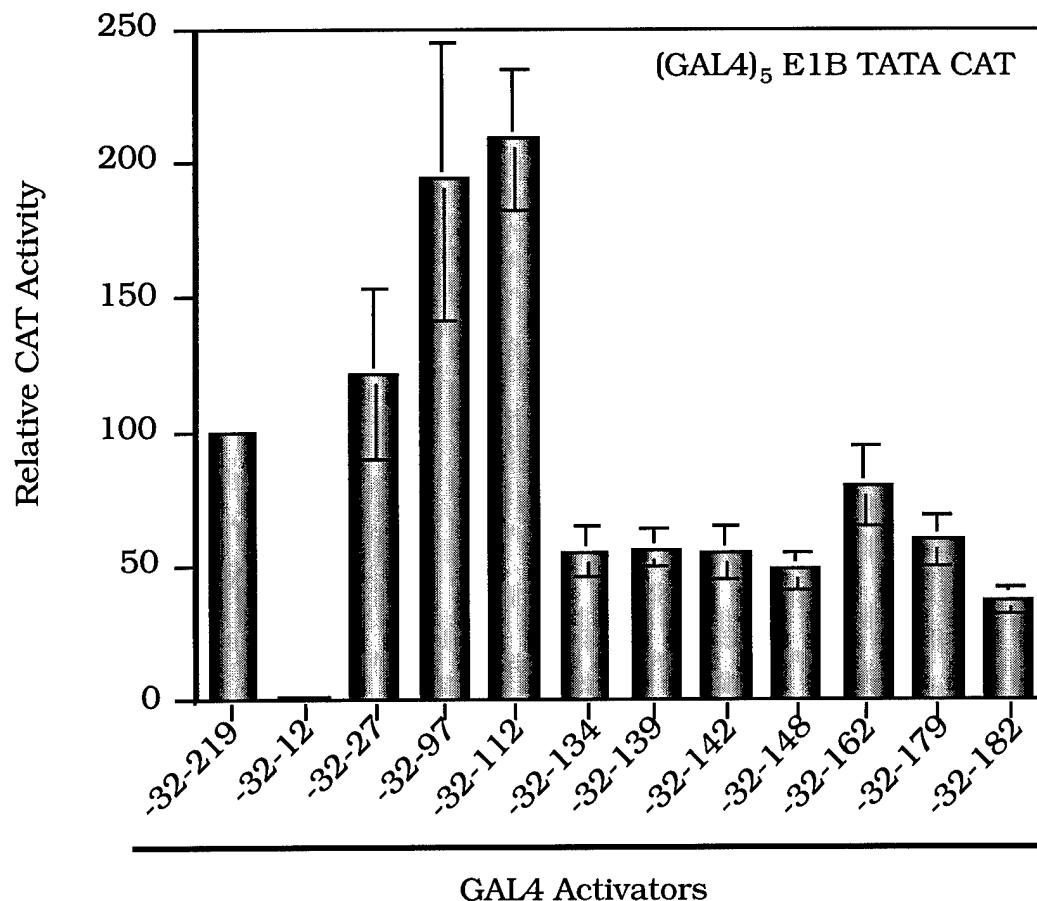


Figure 3.3 Transcriptional activity of the 3' v-Myc TAD deletions. C3H10T1/2 cells were transiently transfected with 5 μ g of each GAL4-v-Myc deletion (Figure 3.1), 5 μ g of the (GAL4)₅ E1B TATA CAT reporter, and 5 μ g of RSV-LacZ to control for transfection efficiency. CAT activity was determined as outlined in Materials and Methods. The relative CAT activity of each GAL4-v-Myc protein is expressed as a percentage of the positive control, GAL4-v-Myc(-32-219), which is set at 100%.

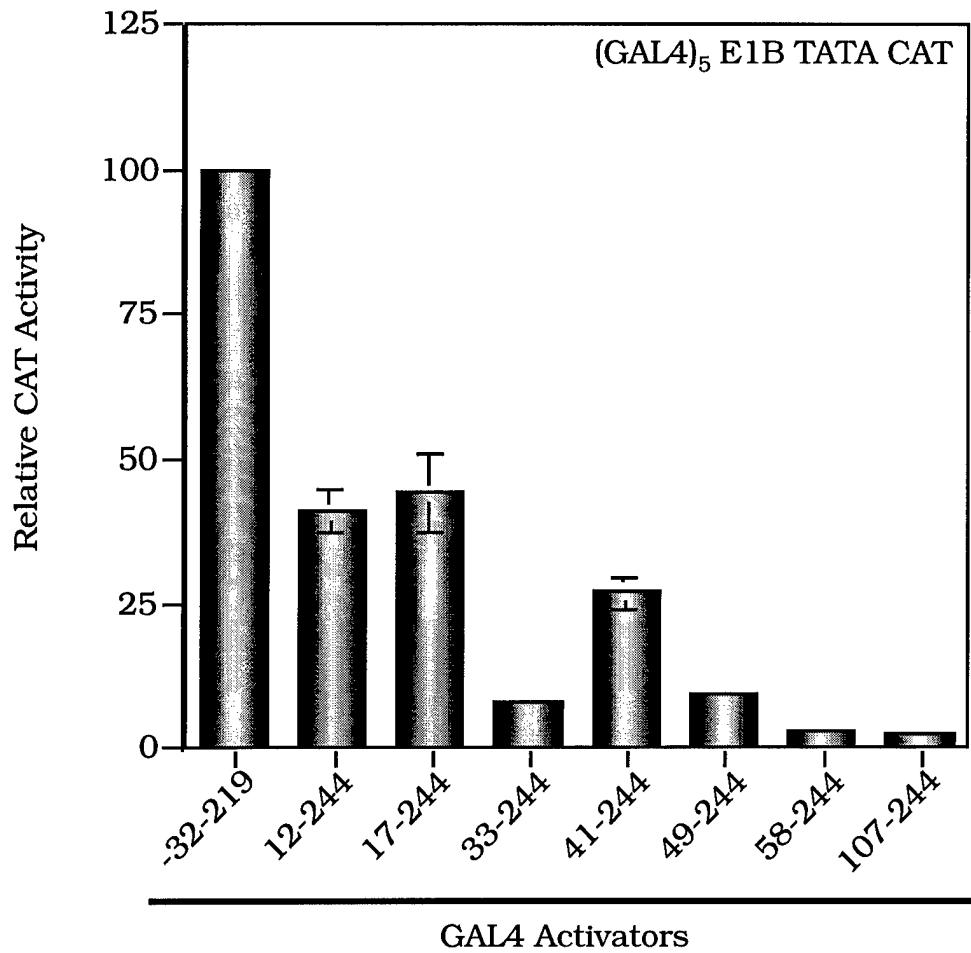


Figure 3.4 Transcriptional activity of the 5' v-Myc TAD deletions. C3H10T1/2 cells were transiently transfected with 5 μ g of each GAL4-v-Myc deletion (Figure 3.2), 5 μ g of the (GAL4)₅ E1B TATA CAT reporter, and 5 μ g of RSV-LacZ to control for transfection efficiency. CAT activity was determined as outlined in Materials and Methods. The relative CAT activity of each GAL4-v-Myc protein is expressed as a percentage of the positive control, GAL4-v-Myc(-32-219), which is set at 100%.

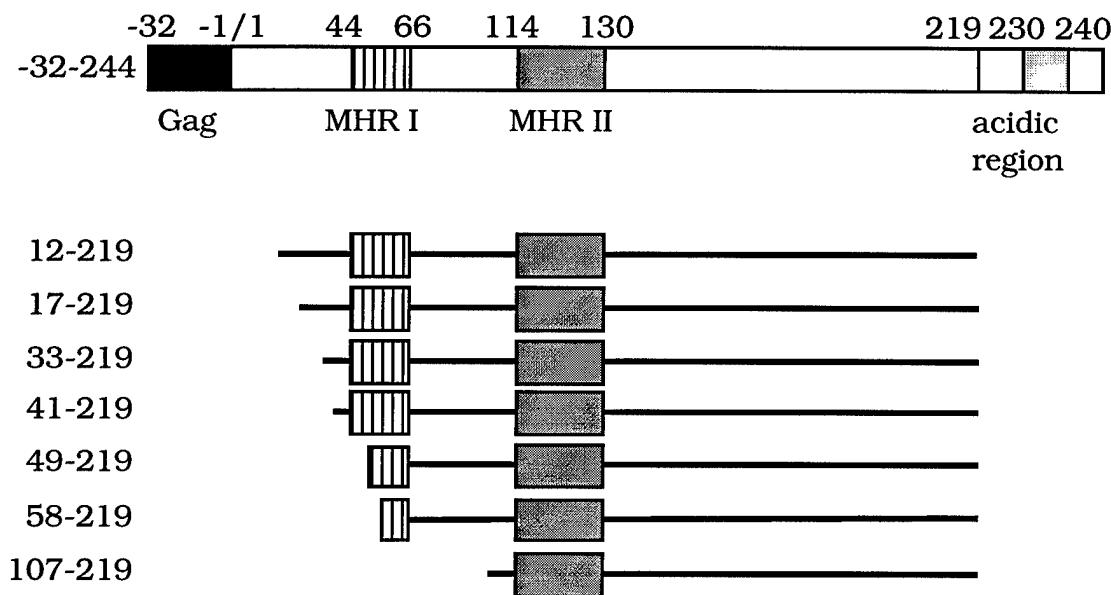


Figure 3.5 Schematic diagram of the modified 5' deletions of the v-Myc TAD. The modified v-Myc 5' mutants each terminate at residue 219 versus the 5' deletions depicted in **Figure 3.2**, which terminate at residue 244. Pictured are the protein sequences remaining in each construct. Each v-Myc protein is fused at the amino terminus to the GAL4 DNA binding domain. Details on the construction of these v-Myc TAD deletions can be found in the Materials and Methods.

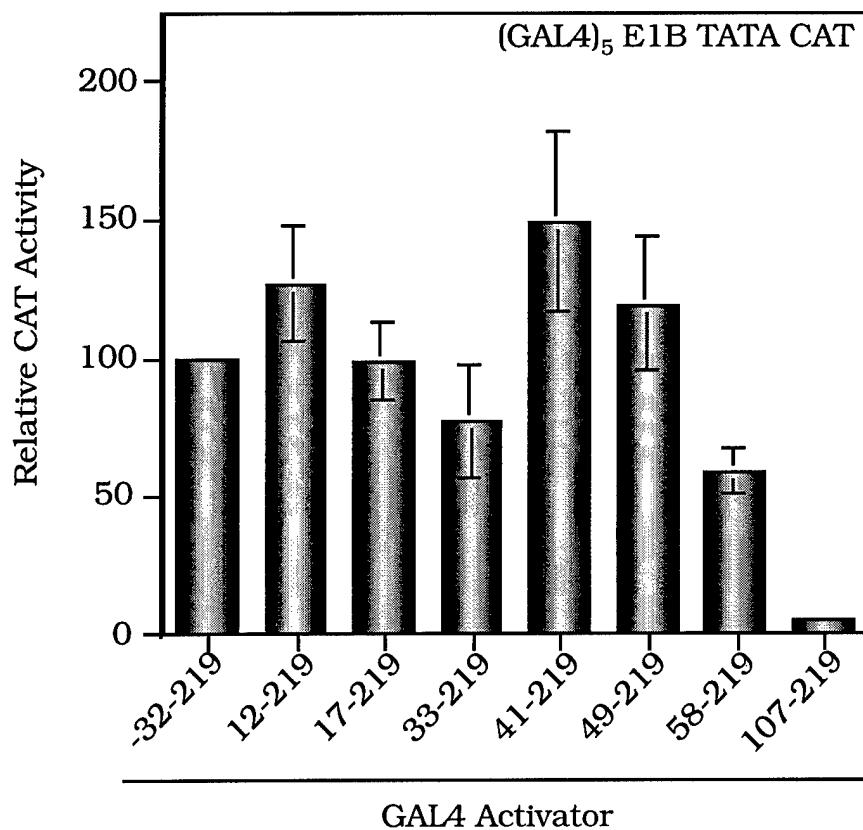


Figure 3.6 Transcriptional activity of the modified 5' v-Myc TAD deletions. C3H10T1/2 cells were transiently transfected with 5 μ g of each GAL4-v-Myc deletion (Figure 3.5), 5 μ g of the (GAL4)₅ E1B TATA CAT reporter, and 5 μ g of RSV-LacZ to control for transfection efficiency. CAT activity was determined as outlined in Materials and Methods. The relative CAT activity of each GAL4-v-Myc protein is expressed as a percentage of the positive control, GAL4-v-Myc(-32-219), which is set at 100%.

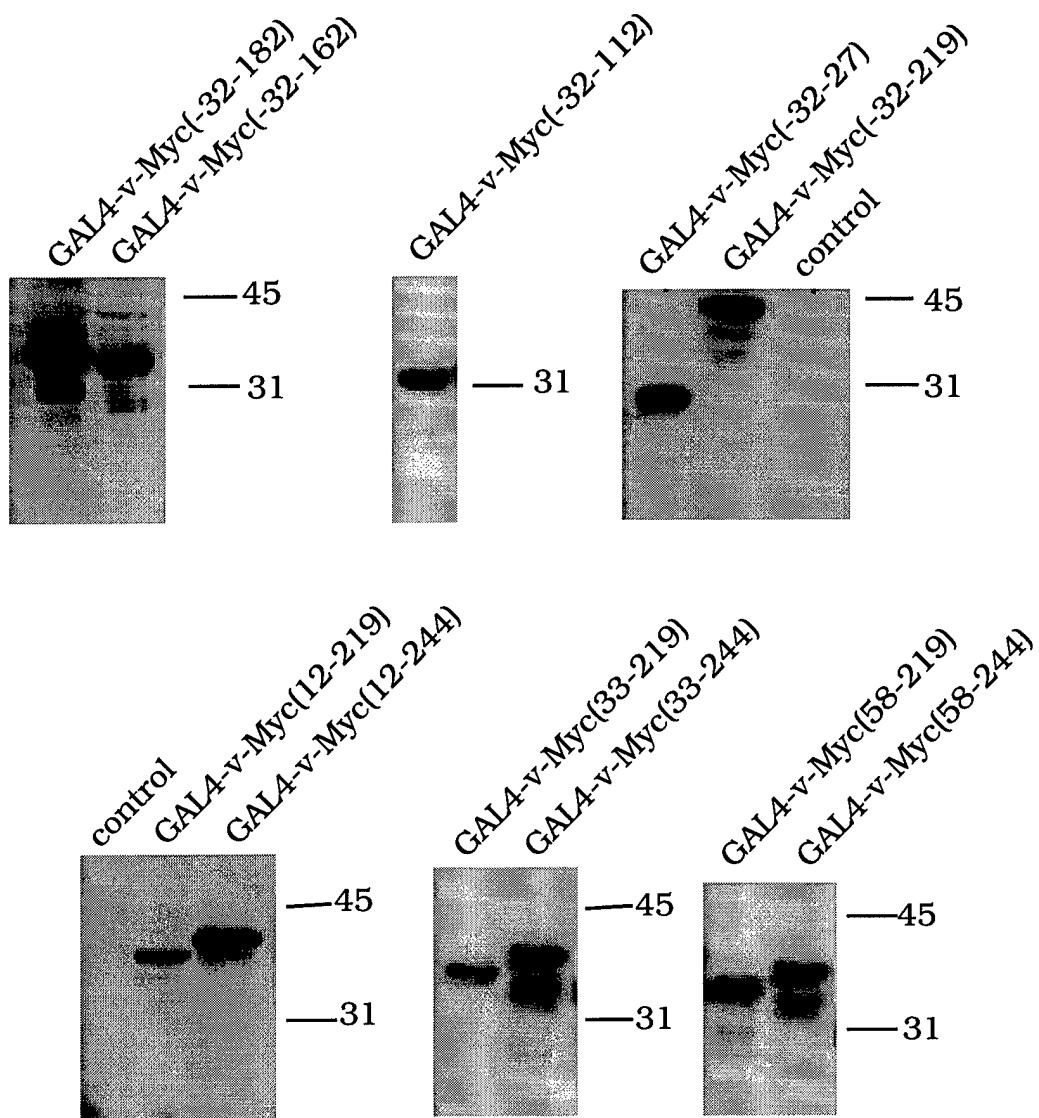


Figure 3.7 Protein expression of selected GAL4-v-Myc fusion proteins. Whole cell extracts of COS-1 cells transiently transfected with 10 μ g of the designated plasmid were prepared, separated by SDS-PAGE, transferred to nitrocellulose, and incubated with an anti-GAL4 antibody as described in the Materials and Methods. ECL detection demonstrates the high level of expression of each of these fusion proteins *in vivo*. The relative migration of molecular weight standards is indicated to the right of each blot.

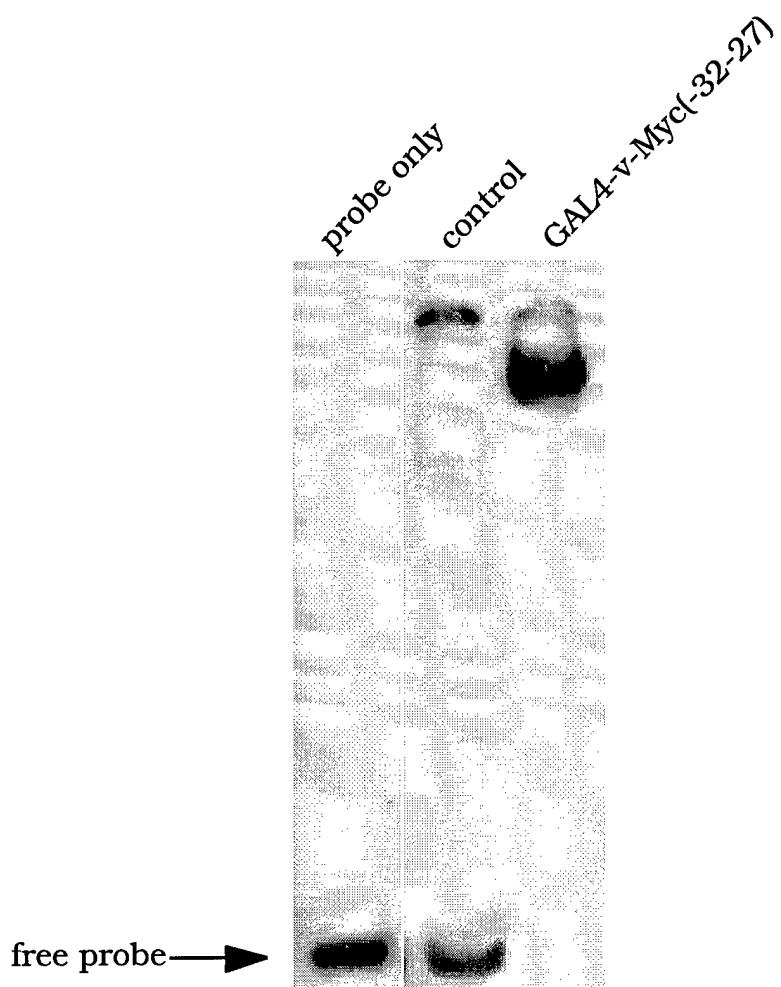


Figure 3.8 DNA binding ability of a selected 3' deletion of Myc, GAL4-v-Myc(-32-27). Nuclear extracts of COS-1 cells transiently transfected with 10 μ g of the designated plasmid were prepared, and 5 μ g of protein from the nuclear extract was incubated with a 32 P-labeled probe consisting of five GAL4 protein binding sites. Samples were separated electrophoretically on a 5% non-denaturing polyacrylamide gel as described in Materials and Methods. The arrow indicates the free probe in the samples. Too little probe was used in this experiment, so no free probe is observed in the GAL4-v-Myc(-32-27) lane.

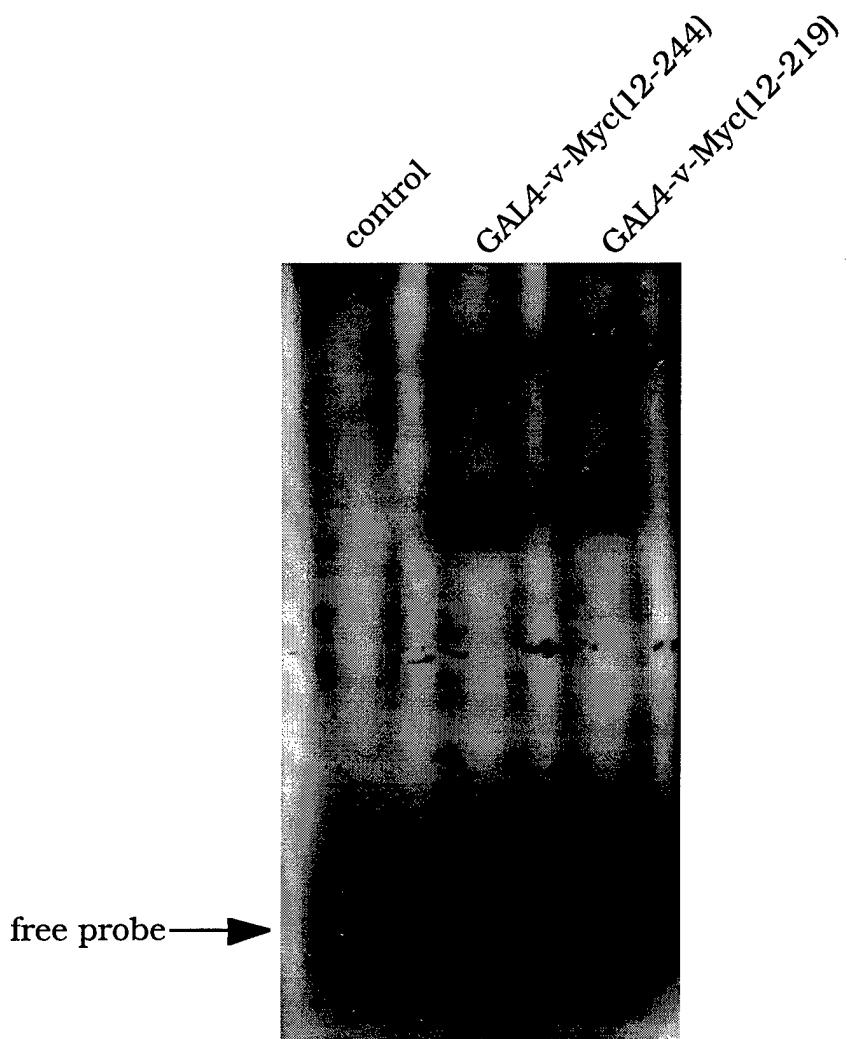


Figure 3.9 DNA binding ability of selected 5' deletions of Myc, GAL4-v-Myc(12-244) and GAL4-v-Myc(12-219). Nuclear extracts of COS-1 cells transiently transfected with 10 μ g of the designated plasmids were prepared, and 5 μ g of protein from the nuclear extracts was incubated with a 32 P-labeled probe consisting of five GAL4 protein binding sites. Samples were separated electrophoretically on a 5% non-denaturing polyacrylamide gel as described in Materials and Methods. The arrow indicates the free probe in the samples.

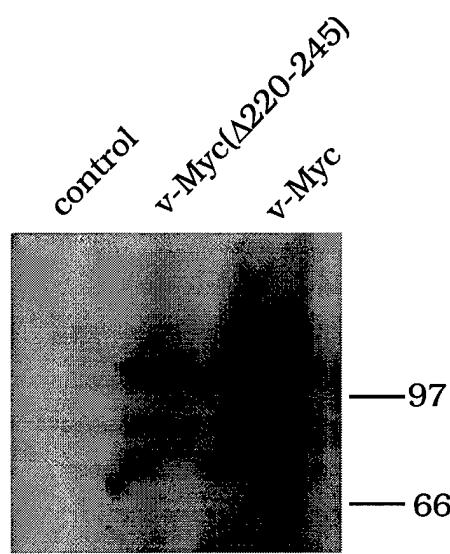


Figure 3.10 Protein expression of full-length and mutant v-Myc proteins. Whole cell extracts of COS-1 cells transiently transfected with 10 μ g of the designated plasmid were prepared, separated electrophoretically by SDS-PAGE, transferred to nitrocellulose, and incubated with an anti-Gag antibody as described in the Materials and Methods. ECL detection demonstrates a high level of expression of these proteins *in vivo*. Molecular weight standards are shown at the side of the blot.

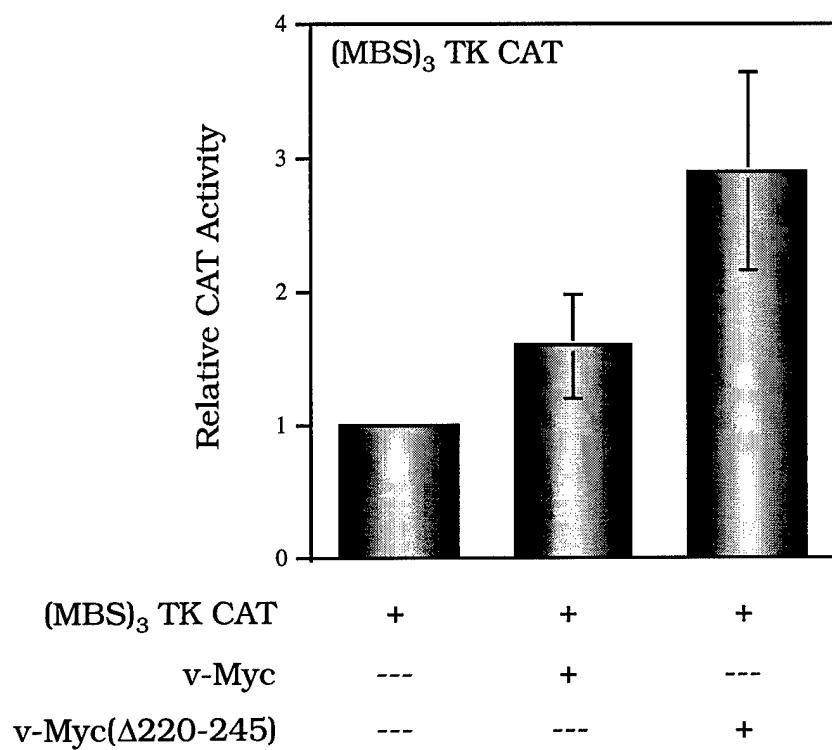


Figure 3.11 Transcriptional activity of full-length and mutant v-Myc proteins. C3H10T1/2 cells were transiently transfected with 5 μ g of the Myc-responsive reporter (MBS)₃ TK CAT and 12.5 μ g of each v-Myc plasmid. CAT activity was determined as outlined in Materials and Methods. The relative CAT activity of each v-Myc protein is expressed as a fold increase over the basal activity of the reporter alone, which is set at 1.0.

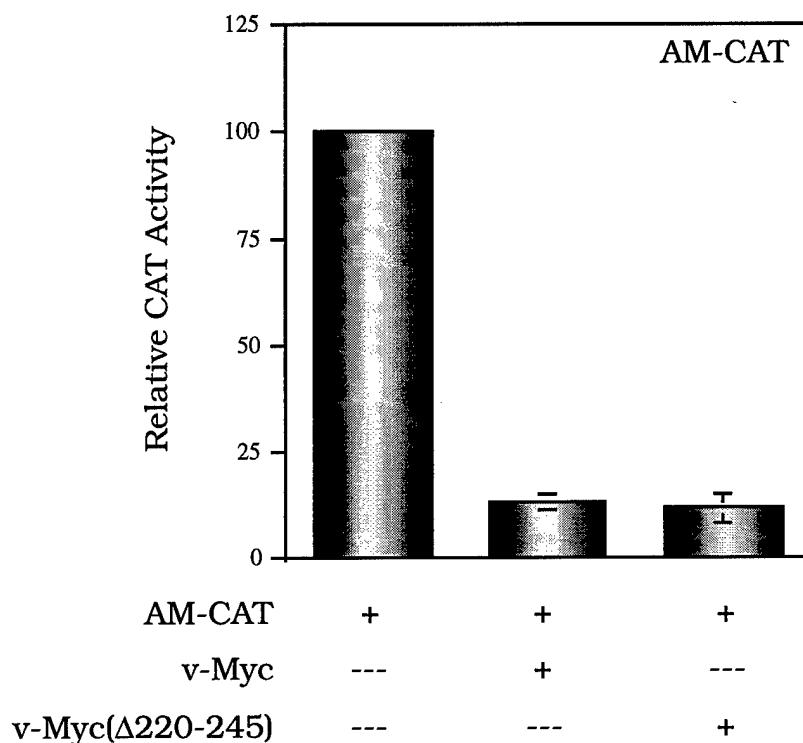


Figure 3.12 Transcriptional repression by full-length and mutant v-Myc proteins. C3H10T1/2 cells were transiently transfected with 2.5 μ g of the Myc-responsive reporter AM-CAT and 5 μ g of each v-Myc plasmid. CAT activity was determined as outlined in Materials and Methods. The CAT activity remaining after co-expression of the reporter and v-Myc protein is expressed as a percentage of the level of CAT activity obtained from the AM-CAT reporter alone, which is set at 100%.

Group	Efficiency of Focus Formation
H-Ras	17% (3)
H-Ras + v-Myc	100% (3)
H-Ras + v-Myc(Δ 220-245)	131% (3)

Figure 3.13 Transforming abilities of full-length and mutant v-Myc proteins with a cooperating activated human H-Ras oncogene. C3H10T1/2 cells were stably transfected with 200 ng pT24 H-ras plus or minus 600 ng pMC29 v-myc or pMC29 v-myc(Δ 220-245). After 14 days of growth in reduced serum medium, cells were fixed, stained, and examined for cellular transformation as described in Materials and Methods. The level of focus formation by H-Ras and v-Myc is set at 100%, and the level of focus formation obtained in other groups is expressed as a percentage of that value. The value in parentheses indicates the number of trials performed for each experimental group.

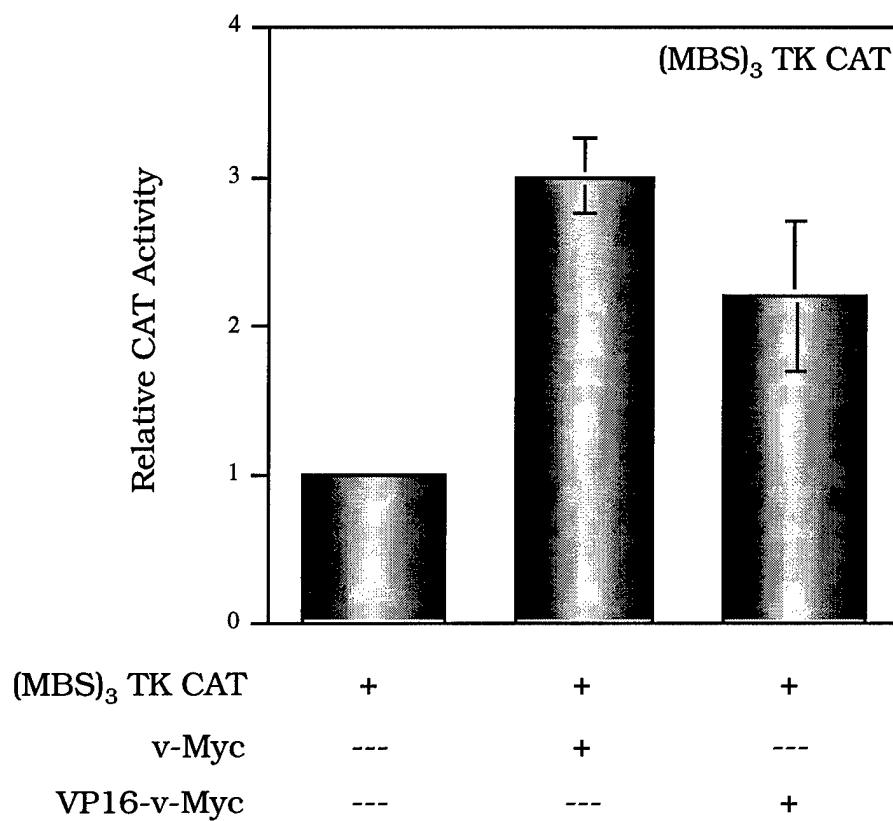


Figure 3.14 Transcriptional activity of wild-type v-Myc and the VP16-v-Myc fusion protein. C3H10T1/2 cells were transiently transfected with 5 μ g of the (MBS)₃ TK CAT reporter and 10 μ g of each v-Myc protein. CAT activity was determined as described in Materials and Methods. The relative CAT activity of each v-Myc protein is expressed as a fold increase over the basal activity of the reporter alone, which is set at 1.0.

Group	Efficiency of Focus Formation
v-Myc	0% (2)
H-Ras	42% (3)
H-Ras + v-Myc	100% (3)
VP16-v-Myc	0% (2)
H-Ras + VP16-v-Myc	29% (3)

Figure 3.15 Transforming abilities of wild-type and the VP16-v-Myc fusion protein with the activated human H-Ras oncogene. C3H10T1/2 cells were stably transfected with 200 ng pT24 H-ras and/or 600 ng pMC29 v-myc or VP-16-v-Myc. After 14 days of growth in reduced serum medium, cells were fixed, stained, and examined for cellular transformation as described in Materials and Methods. The number of foci formed by cooperation of H-Ras and wild-type v-Myc is set at 100%, and the level of focus formation obtained in other groups is expressed as a percentage of that value. The number in parentheses indicates the number of trials performed for each experimental group.

SECTION 4: CHARACTERIZATION OF THE INTERACTION BETWEEN THE V-MYC TAD AND OS-9

Introduction

The many functions of the Myc protein depend absolutely upon the integrity of the Myc amino terminus. Besides this, the presence of two highly conserved regions within the TAD of all Myc proteins suggests that these sequences within the TAD are especially important to Myc protein function. To this end, many proteins have been identified that interact with the Myc amino terminus, presumably to modulate Myc function. Due to evidence generated in our laboratory indicating that the TAD of v-Myc has a unique function in cellular transformation that is distinct from transcriptional activation, a former postdoctoral fellow in the lab, Dr. Michael Dorsey, utilized the yeast two-hybrid screening system (Fields and Song, 1989; Durfee *et al.*, 1993) to identify novel, functionally relevant interacting factors of Myc.

Using the highly conserved MHR II region of the v-Myc TAD (Figure 1.2) as a bait to identify associating partners of v-Myc, an EBV-stimulated B cell cDNA library was screened. From approximately 2.2×10^6 transformants, 22 clones were identified that interacted strongly with v-Myc MHR II but not with control proteins including v-Myc MHR I or p53. Of the 22 positive clones, eight represented overlapping cDNAs that were isolated independently and code for a protein identified via a database search as OS-9 (Dorsey *et al.*, in prep). The *OS-9* cDNA was initially isolated as representing the mRNA of a gene in the region of human chromosome 12q13-15 that is frequently amplified in malignancies (Su *et al.*, 1996). The region encoding the *OS-9* mRNA was isolated from the osteosarcoma cell line OsA-C1 and has been shown to be amplified in 60% of all osteosarcoma cell lines tested (Su *et al.*, 1996). OS-9 is a protein of 667 amino acids with a potential signal sequence at the amino terminus of the protein and overlapping bipartite and SV40-type nuclear localization signals (reviewed in Boulikas, 1994) in the carboxy-terminal portion of the protein. Northern blot analysis of polyadenylated mRNA from several human tissues (Human Multiple Tissue Northern Blots, Clonetech) using a 1.2 kb DNA probe that encodes OS-9 residues 141-561 indicates ubiquitous expression of *OS-9* mRNA in all human tissues examined (Dorsey *et al.*, in prep). Also, Northern blot experiments utilizing *OS-9* and *c-myc* DNA probes demonstrate independent regulation of these two messenger RNAs in all of the human tissues examined. That is, expression of either *OS-9* or *c-myc* does not necessarily correlate with expression of the other (Dorsey *et al.*, in prep). Indirect immunofluorescence experiments of COS-7 cells transiently transfected with hemagglutinin

(HA)-tagged full-length OS-9 protein (residues 1-667) or truncated HA-tagged OS-9 protein (residues 296-667) to determine the cellular location of OS-9 indicates that while the full-length OS-9 protein primarily localizes to the cytoplasm of cells in a punctate staining pattern, the truncated form of OS-9 is nuclear (Dorsey *et al.*, in prep). Complete exclusion from the nucleus of the full-length OS-9 protein cannot be determined, and the translocation of the truncated OS-9 to the cell nucleus after removal of the potential signal sequence argues that the consensus NLS in OS-9 is functional.

To begin to examine the potential for functional interaction between OS-9 and v-Myc, GST pull-down assays were performed with bacterially expressed GST-v-Myc fusion proteins and *in vitro* transcribed and translated OS-9 (residues 424-667) protein (Dorsey *et al.*, in prep). The *in vitro* binding experiments indicate that OS-9 protein can interact only with portions of v-Myc that contain MHR II, the region of v-Myc used in the original yeast two-hybrid screen. Specifically, OS-9 interacts with GST-v-Myc(-32-244) [the full-length v-Myc TAD], GST-v-Myc(90-219) [a portion of the v-Myc TAD that contains MHR II], and GST-v-Myc(114-130) [v-Myc MHR II]. OS-9 does not interact with the GST protein alone, nor does it interact with GST-v-Myc(-32-42), a region of v-Myc that does not encompass MHR II. These *in vitro* binding experiments demonstrate that OS-9 interacts specifically with v-Myc MHR II, which agrees with the yeast two-hybrid data generated previously (Dorsey *et al.*, in prep). However, the *in vivo* interaction between the v-Myc TAD and OS-9 is important to demonstrate the relevance of this association.

Results

v-Myc MHR II and OS-9 interact in a mammalian two-hybrid assay

To further analyze the interaction between v-Myc and OS-9, experiments to address the *in vivo* association of these two proteins were performed. Initially, a modified version of the two-hybrid system was employed to determine if residues comprising v-Myc MHR II could functionally interact with OS-9 in cells. **Figure 4.1** shows the results of the mammalian two-hybrid assay. As was originally depicted in **Figure 3.6**, GAL4-v-Myc(107-219) does not activate transcription from the reporter gene (GAL4)₅ E1B TATA CAT. v-Myc residues 107-219 encompass a portion of the v-Myc TAD that contains MHR II (**Figure 3.5**). When an excess of VP16-TAD OS-9 is co-expressed with GAL4-v-Myc (107-219) in C3H10T1/2 cells, a low but consistent increase in transcriptional activation of the GAL4 CAT reporter gene is observed. This is presumably due to the interaction of the DNA-bound v-Myc residues with the VP16-TAD OS-9 protein. The presence of the VP16 TAD now tethered to the reporter attracts components of the basal

transcription machinery to increase CAT activity of the reporter two-fold. This positive result suggests the potential for *in vivo* interaction between the v-Myc TAD and OS-9.

v-Myc and OS-9 are not co-immunoprecipitated from COS-1 cells

In an attempt to demonstrate a direct *in vivo* interaction between full-length v-Myc and full-length OS-9, COS-1 cells were transiently transfected with either pMC29 *v-myc*, OS-9(1-667), or both. To ensure that the antibodies to be used in the co-immunoprecipitation procedure were able to detect the appropriate proteins when those proteins were expressed either alone or with another factor, Western blot analysis was performed (**Figure 4.2**). Evident in the left panel of **Figure 4.2** is the recognition by the anti-Gag antibody (see Materials and Methods) of the 110 kDa v-Myc protein in lysates from cells transfected with v-Myc and OS-9(1-667). In the right panel of **Figure 4.2** lysates blotted with the anti-HA antibody (Boehringer Mannheim) show expression of the 90 kDa OS-9(1-667) protein in cells transfected with OS-9 or co-transfected with OS-9 and v-Myc. These results demonstrate that the antiserum should be functional in the co-immunoprecipitation experiments.

To assess the ability of v-Myc to interact with OS-9 *in vivo*, COS-1 cells transiently transfected with the desired plasmids were metabolically labeled with Trans-³⁵S Label (ICN) as outlined in the Materials and Methods. In this experiment, two plasmids encoding two distinct OS-9 proteins were used. Full-length OS-9(1-667) was tested, along with the truncated OS-9(296-667) which is localized to the nucleus of cells (see the Introduction of this Chapter). Since Myc is a nuclear protein (Eisenman *et al.*, 1985) it is assumed that Myc would interact with OS-9 in the nuclear compartment and therefore show enhanced binding to a nuclear OS-9. The proteins detected as shown in **Figure 4.3** were precipitated from cell extracts using an anti-HA antibody conjugated to Protein A beads, washed, released from the beads by heat, and separated by SDS-PAGE (see Materials and Methods). Overnight exposure to X-ray film indicates that both the full-length OS-9(1-667) and truncated OS-9(296-667) can be precipitated from the COS-1 cell extracts. The arrows indicate the positions of these precipitated proteins (**Figure 4.3**). However, due to extensive background bands in the area of the gel in which the 110 kDa v-Myc protein should be evident, co-immunoprecipitation of v-Myc cannot be determined using this procedure.

To correct this problem, the sensitivity and low background of antibody detection via Western blot analysis was employed. COS-1 cells transiently transfected with pMC29 *v-myc*, OS-9(1-667), or both DNAs were harvested and incubated with anti-HA antibody conjugated to Protein A beads as described in the Materials and Methods. These extracts were separated by SDS-

PAGE, transferred to nitrocellulose, and incubated with an anti-Gag antibody to detect the presence of the v-Myc protein. As barely visible in **Figure 4.4**, a band of 110 kDa was detected in the sample obtained from cultures expressing both v-Myc and OS-9(1-667). Unfortunately, the same band at the same intensity was evident in the sample from cells transfected with v-Myc alone, indicating that this faint band represents a non-specific interaction of the anti-Gag antibody. Since several different attempts to co-immunoprecipitate v-Myc and OS-9 from cells have failed, other avenues to establish an indirect interaction between the two proteins will be explored in the future.

Discussion

The involvement of Myc in multiple protein-protein interactions represents a mechanism by which Myc function is modulated in cells. For example, many studies have demonstrated that Myc can activate transcription from responsive promoters. Not surprisingly, the Myc TAD interacts with the TATA-binding protein (TBP) of the TFIID transcription initiation complex (Maheswaran *et al.*, 1994). This association serves to explain the observed activation of transcription resulting from Myc expression. On the other hand, c-Myc also associates with α -tubulin and polymerized microtubules in the cytoplasm of cells (Alexandrova *et al.*, 1995). Despite the characterization of Myc as a predominantly nuclear protein (Eisenman *et al.*, 1985), increased levels of cytoplasmic Myc protein are associated with cessation of proliferation in some cells. For example, c-Myc in proliferating NIH 3T3 cells localizes primarily to the nucleus, while cells that have ceased to grow due to contact inhibition exhibit a shift from nuclear-localized c-Myc to cytoplasmically-localized c-Myc (Vriz *et al.*, 1992). Supporting these data is the observation that during differentiation of human myeloid leukemia cells there is a translocation of c-Myc from the nucleus to the cytoplasm of the cells (Craig *et al.*, 1993). This implies that during conditions of cellular growth arrest, c-Myc is stored or sequestered in the cytoplasm, perhaps through interaction with a cytoplasmic protein like α -tubulin.

Considering that Myc's functions are attributed to its role as a nuclear transcription factor, it is surprising that predominantly cytoplasmic proteins like OS-9 would interact with Myc. However, **Figure 4.1** suggests that an *in vivo* interaction between v-Myc and OS-9 does take place. The mammalian two-hybrid assay has been used previously to demonstrate the interaction between the highly studied muscle-specific transcription factor myogenin and products of the E2A gene (Chakraborty *et al.*, 1992). Although the increase in CAT activity between v-Myc and OS-9 was much less than the published increase in activity between myogenin and E12 or E47 in

this same assay system (2-fold versus 80-fold, respectively), the two-fold increase in activity was reproducible and statistically significant (**Figure 4.1**).

The cross-reactivity of the antibodies that led to detection of v-Myc with the anti-HA antibody in **Figure 4.4** was disappointing and puzzling. When either the anti-Gag or anti-HA antibody was used for Western blotting extracts from the same experimental groups, very little background was observed (**Figure 4.2**). This suggests that the antibodies are specific, at least in a Western blot. Perhaps the longer incubation time of antibody and sample used during the co-immunoprecipitation protocol allows for increased non-specific interaction. While unlikely, addition of beads to the cell extracts could perhaps also alter protein precipitation. Another puzzling fact is the inability of the anti-Gag antibody to immunoprecipitate the v-Myc protein from labeled COS-1 cells (data not shown). This limited the range of reagents useable for the experiments.

Since both the OS-9(1-667) and OS-9(296-667) were precipitated efficiently from the labeled cell extracts by the anti-HA antibody (**Figure 4.3**), it was assumed that binding conditions were adequate to retain the v-Myc/OS-9 interaction. In fact, different detergent concentrations were utilized to decrease background but to maintain the OS-9 interaction. The failure to co-immunoprecipitate v-Myc and OS-9 from cells does not mean that the two proteins do not interact *in vivo*. On the contrary, data presented in **Figure 4.1** and in Chapter 5 suggests that the two proteins do indeed associate, at least functionally. Perhaps the physical interaction between v-Myc and OS-9 is very transient and therefore difficult to maintain during the rigorous manipulations of a co-immunoprecipitation. OS-9 does not contain traditional protein interaction motifs such as a helix-loop-helix (Murre *et al.*, 1989) or a leucine zipper (Landschulz *et al.*, 1988). OS-9 does, however, contain two regions 44 amino acid in length that are imperfect direct repeats of one another (Dorsey *et al.*, in prep) homologous to the tandem repeats of the Ski oncoprotein that are responsible for protein homo- and heterodimerization (Heyman and Stavnezer, 1994). However, no such repeats of amino acids occur in Myc, so it is unlikely that these motifs in OS-9 mediate the association of v-Myc MHR II and OS-9. With no structurally defined dimerization interface, OS-9 and v-Myc most likely rely upon ionic charge or hydrophobic residues (Pabo and Sauer, 1992) to provide an appropriate surface for interaction. These types of interactions may be weak and refractile to analysis by the techniques used in this study. Future efforts will be geared toward using gel overlays to continue investigating the potential of OS-9 and v-Myc to interact physically in some capacity.

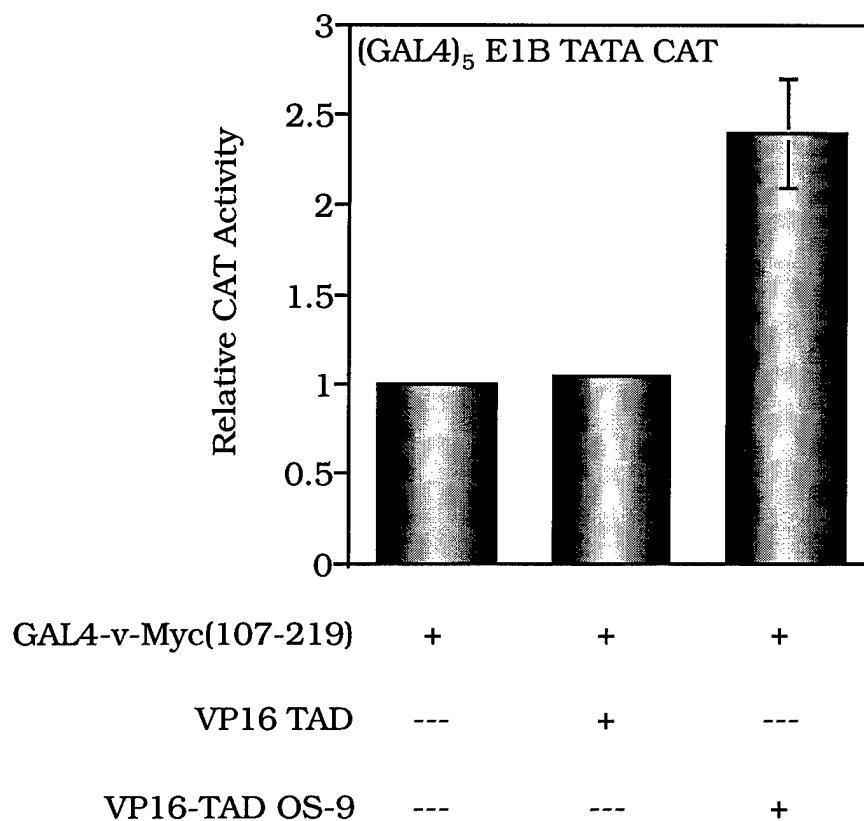


Figure 4.1 Interaction of v-Myc MHR II and OS-9 activates transcription in a mammalian two-hybrid assay. C3H10T1/2 cells were transiently transfected with 5 μ g of the (GAL4)₅ E1B TATA CAT reporter, 1 μ g of GAL4-v-Myc(107-219), 10 μ g of VP16-TAD-OS-9, and 5 μ g of RSV-LacZ to control for transfection efficiency. CAT activity was determined as outlined in Materials and Methods. The average CAT activity of GAL4-v-Myc(107-219) is set at 1.0, and the activity resulting from the interaction of GAL4-v-Myc(107-219) and VP16-TAD-OS-9 is expressed as a fold increase over this value.

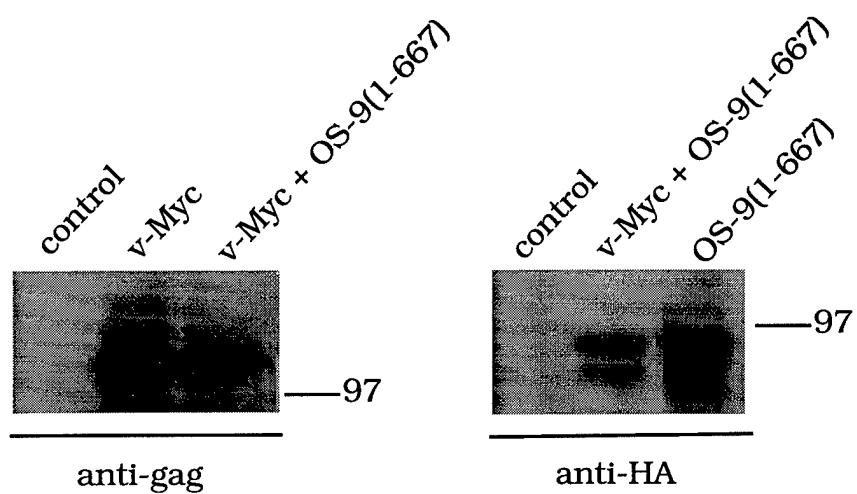


Figure 4.2 Western blot analysis of proteins used in the co-immunoprecipitation experiments. Whole cell extracts of COS-1 cells transiently transfected with 10 μ g of either pMC29 *v-myc*, *OS-9(1-667)*, or both plasmids were prepared, separated by SDS-PAGE, transferred to nitrocellulose, and incubated with either an anti-Gag or anti-HA antibody as described in the Materials and Methods. ECL demonstrates that each antibody detects the appropriate protein when expressed alone or in combination with the other protein. A molecular weight standard is indicated to the right of each blot.

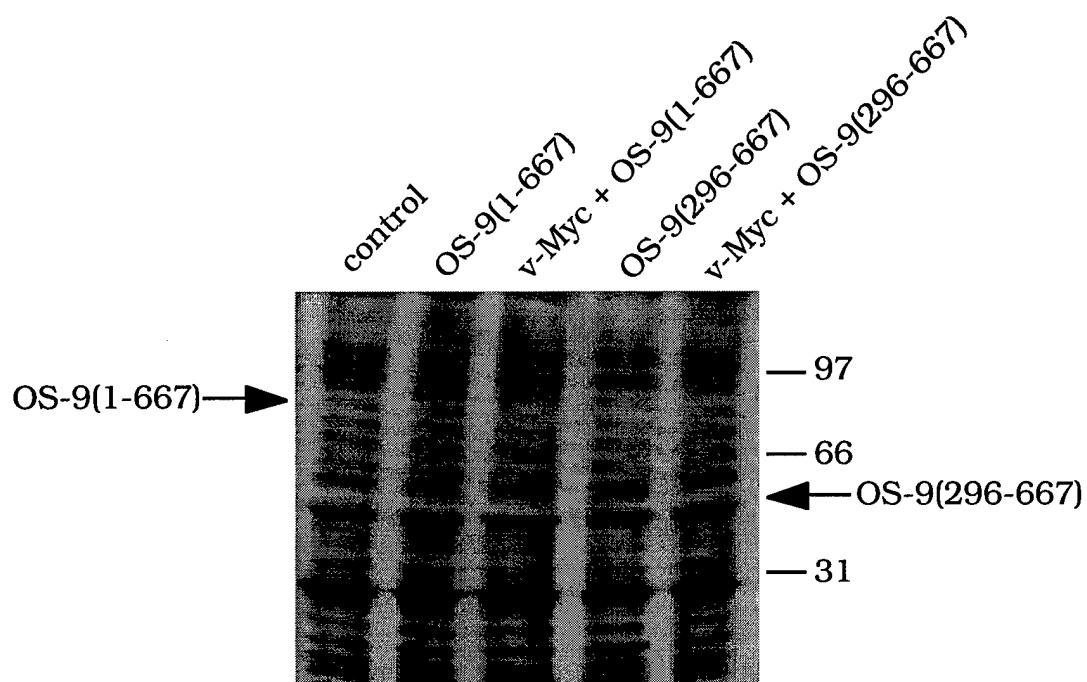


Figure 4.3 Immunoprecipitation of HA-tagged OS-9 proteins from ^{35}S -methionine labeled COS-1 cells. COS-1 cells were transiently transfected with 20 μg of the indicated plasmid and 48 hours post transfection metabolically labeled with ^{35}S -methionine as described in Materials and Methods. HA-tagged OS-9 proteins and any interacting factors were immunoprecipitated using an anti-HA antibody conjugated to Protein A beads. Samples were separated electrophoretically by SDS-PAGE, dried, and exposed to X-ray film. Molecular weight standards are indicated on the right of the autoradiograph. Arrows indicate the positions of the precipitated full-length and truncated OS-9 proteins.

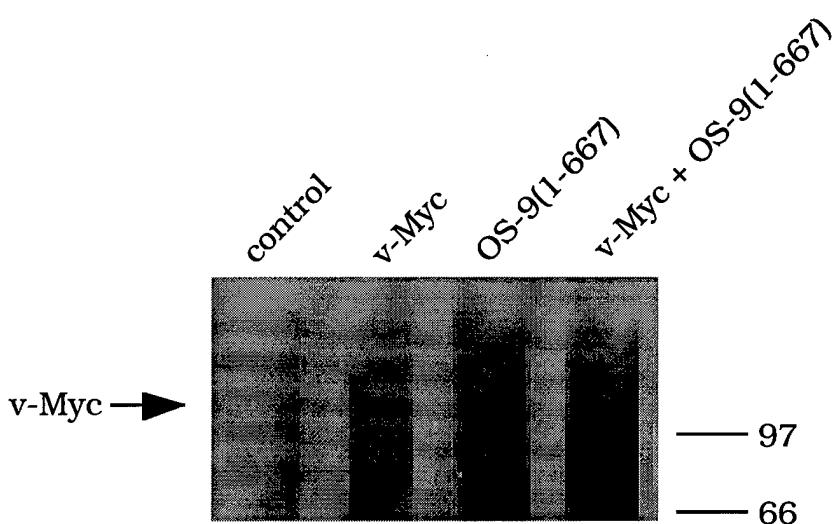


Figure 4.4 Co-immunoprecipitation of v-Myc and OS-9 from non-labeled cells. COS-1 cells were transiently transfected with 10 μ g of the indicated plasmids. After 48 hours, cell lysates were prepared and incubated with anti-HA antibody conjugated to Protein A agarose beads as described in Materials and Methods. Samples were released from the beads by heat and separated by SDS-PAGE, transferred to nitrocellulose, and incubated with the anti-Gag antibody to detect the presence of v-Myc (see Materials and Methods). Molecular weight markers are indicated to the right of the autoradiograph. The position of a co-immunoprecipitated 110 kDa protein in the v-Myc + OS-9(1-667) as well as the v-Myc only samples is indicated by the arrow.

SECTION 5: IMPACT OF OS-9 ON v-MYC FUNCTION

Introduction

The ability of Myc to interact with cellular proteins undoubtedly is a means to modulate Myc function. Association between Myc and the TATA binding protein TBP of the TFIID transcription initiation complex allows the Myc protein to bring about activated transcription of Myc-responsive promoters (Hateboer *et al.*, 1993; Maheswaran *et al.*, 1994). Additionally, the retinoblastoma-related protein p107 has been shown to interact specifically with the amino terminus of c-Myc *in vivo* (Beijersbergen *et al.*, 1994; Gu *et al.*, 1994). Interestingly, this association of Myc and p107 results in inhibition of Myc transcriptional activity. More important clinically, many Burkitt's and AIDS-related lymphomas contain point mutations within the Myc transcription activation domain. These mutant Myc proteins no longer interact physically with p107, and this results in enhanced transcriptional activation by c-Myc. Since Myc activity is no longer attenuated by p107 in these lymphomas, this is advantageous to the growth of the lymphoma cells (Hoang *et al.*, 1995).

The identification of OS-9 as a v-Myc MHR II-interacting factor (Dorsey *et al.*, in prep) is important as it may reveal another mechanism by which Myc activity is modulated *in vivo*. Since the OS-9/Myc association is mediated by the amino terminus of Myc, the functions coded for by this region of Myc would most likely be influenced by OS-9. The amino terminus of Myc is absolutely required for Myc-mediated cellular transformation, transcriptional activation/repression by Myc, and induction of apoptosis. Because *OS-9* has been identified as a gene residing within a region of human chromosome 12 often amplified in sarcomas (Su *et al.*, 1996), it is very likely that the OS-9 protein offers some type of growth advantage to cells in which it is overexpressed. The association with Myc and the possible influence on Myc function could provide insight into why the *OS-9* locus has been implicated in the development of these tumors.

Results

Full-length or truncated OS-9 proteins do not influence cellular transformation by Ras and v-Myc

Since the integrity of MHR II in the Myc proteins is important for Myc-mediated cellular transformation (Stone *et al.*, 1987; Brough *et al.*, 1995), and because OS-9 interacts with MHR II, the ability of OS-9 to influence cellular transformation events induced by co-expression of activated Ras and v-Myc was examined. C3H10T1/2 cells were stably transfected with 200 ng

pT24 *H-ras* and/or 600 ng pMC29 *v-myc* with or without 2 µg *OS-9(1-667)* or *OS-9(297-667)* and allowed to grow in reduced serum medium (BME + 5% FBS + P/S) for 14 days as described in Materials and Methods. At this point, cellular transformation was visually assessed by counting the number of transformed foci on each plate. As can be ascertained from **Figure 5.1**, neither the predominantly cytoplasmic, full-length OS-9 protein (residues 1-667) or the nuclear-localized, truncated OS-9 protein (296-667) transforms cells on its own. v-Myc expressed in cells also does not generate transformed foci. Expression of activated H-Ras induces a moderate level of focus formation in C3H10T1/2 cells which is unaffected by co-expression of the full-length or truncated OS-9 proteins (**Figure 5.1**). When activated H-Ras and v-Myc are overexpressed *in vivo*, a maximum level of focus formation is achieved, which is set at 100%. However, as observed in Ras-mediated transformation, expression of *OS-9(1-667)* or *OS-9(296-667)* in the presence of H-Ras and v-Myc does not influence significantly the efficiency of focus formation obtained with H-Ras and v-Myc (**Figure 5.1**). This suggests that despite interacting with a region of v-Myc essential for Myc's ability to transform cells, OS-9 does not positively or negatively regulate this function of the Myc protein.

OS-9 represses transcriptional activation by Myc fusion proteins

To determine whether OS-9 affects other functions of the v-Myc amino terminus, transcriptional activation in the presence of overexpressed OS-9 was analyzed. C3H10T1/2 cells were transiently transfected with 5 µg of various GAL4-v-Myc chimeric activators and a two-fold molar excess of *OS-9(1-667)* plasmid DNA. **Figure 5.2** demonstrates that when *OS-9(1-667)* is overexpressed, the transcriptional activities of several GAL4 fusion proteins are inhibited. For example, both forms of the v-Myc TAD (residues -32-219 and -32-244) are repressed by *OS-9(1-667)*, and the corresponding region of the c-Myc TAD (residues 1-262) is similarly repressed. v-Myc residues 90-219 (which contain MHR II) are inhibited by *OS-9(1-667)*, but interestingly, v-Myc residues -32-42 are unaffected. This parallels *in vitro* binding studies which show that OS-9 can interact with v-Myc residues 90-219 but not with -32-42 (Dorsey *et al.*, *in prep*). Also of interest is the repression of transcriptional activation by the TAD of the viral activator protein VP16 in the presence of *OS-9(1-667)*. This supports evidence generated in this laboratory suggesting v-Myc and VP16 interact with common cellular targets necessary for transcriptional activation and cellular transformation (Min *et al.*, 1994). Importantly, the transcriptional activities of not all GAL4 activators tested were affected by *OS-9(1-667)*. **Figure 5.2** indicates that the transcription activation domain of the adenovirus E1A protein (residues 121-223), or of the tissue-specific factor MyoD (residues 1-66), is not significantly influenced by *OS-9(1-667)* overexpression, suggesting that OS-9 does not inhibit transcriptional activation in

general, perhaps by sequestering factors needed for basal transcription (Ptashne and Gann, 1990; Martin, 1991).

Since OS-9(1-667) was determined to be predominantly cytoplasmic by cell staining experiments (Dorsey *et al.*, in prep), the repression of transcriptional activation when OS-9(1-667) is overexpressed may be due to retention of interacting activators in the cytoplasm where they would be unavailable to participate in the events responsible for activation of CAT expression. To address this possibility, the nuclear-localized form of OS-9 (residues 296-667) was used to repeat these experiments. **Figure 5.3** depicts the results of co-expressing the same GAL4 activators used in **Figure 5.2** and OS-9(296-667). The truncated OS-9 protein is similar to the full-length OS-9(1-667) in its ability to repress transcriptional activation. These results imply both the cytoplasmic and nuclear forms of OS-9 inhibit Myc (and VP16) transcriptional activation.

To further analyze this repressive effect, a second reporter gene system was utilized to ensure the observed OS-9 effect was not somehow a reporter-specific phenomenon. The v-Myc TAD fused to the DNA binding domain of the bacterial protein LexA activates the (LexA)₆ E1B TATA CAT reporter, and this activity is repressed to the same extent as seen in the GAL4 system when a two-fold molar excess of either OS-9(1-667) or OS-9(296-667) is co-expressed with LexA-v-Myc(-32-244) (**Figure 5.4**). Therefore, the repression observed in the GAL4 reporter system is also seen in the LexA system which strongly suggests this phenomenon is targeted to the Myc TAD.

OS-9 represses Myc-mediated transcriptional activation of genes regulated by Myc E box sequences

In order for the interaction between v-Myc and OS-9 to be physiologically relevant, OS-9 should have the same effect on the function of the full-length protein as it does on artificial Myc-based activator reporter systems. As a first step toward examining this, the (MBS)₃ TK CAT reporter was utilized. This reporter contains three copies of the Myc E box upstream of a minimal promoter and CAT gene. The high level of basal activity of this reporter in cells (**Figure 5.5**) is most likely due to endogenous c-Myc protein within the cells analyzed, and therefore, expression of OS-9(1-667) does negatively affect the activity of the reporter construct alone (**Figure 5.5**). Interestingly, when this reporter is activated by v-Myc expression, OS-9(1-667) inhibits transcriptional activation to the level of controls. This demonstrates that OS-9 can influence transcriptional activation by full-length Myc proteins.

OS-9 functions as a general transcriptional repressor if tethered to DNA

To address how OS-9 influences Myc-mediated transcriptional activation, a complex reporter consisting of both LexA- and GAL4-protein binding sites was utilized to determine the effect of tethering OS-9 to DNA by the heterologous binding domain of GAL4. **Figure 5.6** illustrates the transactivation properties of several LexA activators on the (LexA)₈ (GAL4)₅ CAT reporter (black bars). Expression of GAL4-OS-9 results in repression of each of these activators (gray bars), even those activators that were not affected by OS-9 expression in the GAL4 experiments [v-Myc(-32-42), E1A(121-223), and MyoD(1-66)]. This repression is specific to OS-9 in that an unrelated protein (the basic leucine zipper transcription factor, B-ATF) does not affect transcriptional activation when tethered to DNA in an identical way (striped bars). This implies that bringing OS-9 into the proximity of a promoter, either by interaction with Myc (or VP16) or by directly binding to DNA, results in inhibition of transcription.

*OS-9 enhances transcriptional repression of *Inr*-containing promoters by Myc*

To investigate whether OS-9 affects other Myc-mediated cellular activities, the ability of OS-9 to influence transcriptional repression by v-Myc was analyzed. Repression of gene expression by c-Myc has been studied primarily using the adenovirus-2 major late promoter (Li *et al.*, 1994). The minimal form of this promoter (pMLb) consists of the adenovirus-2 TATA box and an *Inr* element linked to a luciferase reporter gene. **Figure 5.7** illustrates the down-regulation of this reporter gene by increased v-Myc expression (compare the activity represented by the black bar to activities shown by the shaded bars). As reported previously (Li *et al.*, 1994) overexpression of another basic helix-loop-helix/leucine zipper protein USF (striped bars) activates this reporter in a dose-dependent fashion. Increasing expression of the full-length OS-9 protein, however, has no effect on the activity of the pMLb reporter (white bars). Since the basal level of activity of the reporter gene used in this assay is very low when expressed alone (**Figure 5.7**), USF is co-expressed with the reporter to increase activity prior to measuring repression by Myc (Li *et al.*, 1994). The activity of pMLb-luc plus USF is shown in the gray bar in **Figure 5.8**. As can be seen in **Figure 5.8**, increased expression of v-Myc results in a stepwise reduction of CAT activity of the pMLb-luc reporter (black bars). When OS-9(1-667) is expressed along with USF and v-Myc, however, enhanced transcriptional repression is observed (shaded bars). In these experiments, it appears that OS-9(1-667) makes Myc a more efficient repressor. To revisit this phenomenon using a cellular promoter instead of the adenovirus promoter, the Myc-repressible *adrenomedullin* gene (AM) was used. 2 kb of 5' flanking sequence of the AM gene is linked to the CAT reporter gene to generate AM-CAT (see Materials and Methods) which is down-

regulated following co-expression of v-Myc (Wang *et al.*, submitted). **Figure 5.9** shows the high level of activity of the AM-CAT reporter *in vivo*. Expression of v-Myc results in a 70% reduction of this reporter. As was observed in the case of pMLb-luc, co-expression of OS-9(1-667) further enhances the repression of AM-CAT by v-Myc, leaving only 5% residual CAT activity (**Figure 5.9**). OS-9(1-667) alone does affect the basal activity of AM-CAT, presumably by interfering with the activity of the endogenous c-Myc protein. Interestingly, the ability of OS-9 to enhance repression of AM-CAT by v-Myc is specific, since the unrelated transcription factor B-ATF does not alter the repression of AM-CAT by v-Myc (**Figure 5.9**). To further assess the specificity of this observation, we tested if the unrelated β -actin CAT reporter (Fregien and Davidson, 1986) was influenced by OS-9. This reporter displays a high level of activity when expressed *in vivo* (**Figure 5.10**). However, neither expression of OS-9(1-667), v-Myc, or both proteins significantly reduced levels of β -actin CAT activity in C3H10T1/2 cells. This implies that only physiologically relevant targets of Myc are influenced by OS-9 expression.

The cell line OS-9-5 exhibits reduced growth and apoptosis when compared to Myc neo 13A cells

As a final method to examine the effect of OS-9 on Myc function, a cell line was generated that overexpresses both v-Myc and OS-9(1-667). Myc neo 13A cells (Davenport and Taparowsky, 1992) were stably transfected with OS-9(1-667) in order to generate a cell line that expresses both v-Myc and OS-9 (see Materials and Methods). The clone designated OS-9-5 was chosen due to its high level of expression of the OS-9 protein. **Figure 5.11** shows a Western blot analysis of the parental Myc neo 13A cells and OS-9-5. The left panel demonstrates that only the OS-9-5 cell line expresses the HA-tagged OS-9(1-667) protein, while both cell types express v-Myc (right panel, **Figure 5.11**). The growth properties of the OS-9-5 cell line, Myc neo 13A cells, and the original parent cell line C3H10T1/2 were analyzed by growth curves as shown in **Figure 5.12**. Each cell line was plated out at low density (1×10^4 cells per plate) in multiple 60 mm dishes in BME supplemented with 10%FBS and cell densities determined over a 9-day period (see Material and Methods). The growth curves in **Figure 5.12** indicate that the Myc neo 13A cells continue to proliferate throughout the counting period, whereas the C3H10T1/2 cells reached saturation density at approximately 1×10^6 cells per plate (**Figure 5.12**). Interestingly, OS-9-5 cells do not continue to proliferate as Myc neo 13A cells do and reach a final saturation density twice that of C3H10T1/2 cells. Initially, the growth rates of Myc neo 13A and OS-9-5 cells are similar (days 1-6). However, the OS-9-5 cells then begin to slow growth and eventually reach their final saturation density (**Figure 5.12**).

Since OS-9 seems to attenuate Myc-induced growth in the C3H10T1/2 cells, the ability of OS-9 to influence Myc-mediated apoptosis was analyzed. These studies were performed with the help of Dr. Dorothy Teegarden, Department of Foods and Nutrition, Purdue University, West Lafayette, IN. All three cell lines analyzed in **Figure 5.12** were grown to near-confluence in growth medium (BME + 10%FBS + P/S) and harvested, or grown to near-confluence and changed to starvation medium (BME + 0.5% FBS + P/S) for 48 hours prior to harvesting. Previous experiments from our laboratory (Davenport an Taparowsky, 1992) suggested that under reduced serum conditions, v-Myc-expressing C3H10T1/2 cells undergo apoptosis. Both detached cells and adherent cells of each line in the different conditions were collected and analyzed. The percentage of cells undergoing apoptosis and the stage of the cell cycle were determined by fluorescence activated cell sorting, or FACS (see Materials and Methods). **Figure 5.13** illustrates the analysis of C3H10T1/2 cells in both growth and starvation conditions. The upper panel demonstrates the apoptotic index of the C3H10T1/2 cells in growth medium. The inset box labeled "I" segregates the apoptotic cells from non-apoptotic cells. In this experiment, 0.2% of C3H10T1/2 cells are undergoing apoptosis in BME plus 10%FBS. The lower panel represents C3H10T1/2 cells maintained in starvation medium. In this case, 5.0% of cells are apoptotic, an increase of 25-fold. The v-Myc-overexpressing cell line Myc neo 13A is analyzed in **Figure 5.14**. In growth medium (top panel) 0.4% of cells examined are apoptotic (inset box marked "I"). However, when Myc neo 13A cells are maintained in starvation medium for 48 hours, 30.8% of cells undergo apoptosis (bottom panel), an increase of 77-fold. OS-9-5 cells were analyzed in **Figure 5.15**. While a greater number of cells in growth conditions were apoptotic (8.7%; upper panel), only a negligible increase in cellular apoptosis was detected when OS-9-5 cells were grown in starvation medium (11.4%; lower panel). This translates into a 1.3-fold increase in apoptosis in OS-9-5 cells grown in 0.5% FBS versus OS-9-5 cells maintained in 10% FBS. This indicates that besides altering the overall proliferative capacity of cells overexpressing v-Myc, OS-9 also attenuates the apoptotic response of Myc-expressing cells.

Discussion

OS-9 was first identified as a cDNA representing mRNA transcribed from a gene within a region of the long arm of human chromosome 12 that is amplified frequently in osteosarcoma cell lines (Su *et al.*, 1996). Further analysis of this region of chromosome 12q13.3 indicates that five genes (*OS-9*, *CDK4*, *SAS*, *KIA0167*, and *6E5.2*) are transcribed when this region of the chromosome is amplified. This extremely gene-dense region makes it difficult to determine which genes are primary amplicons and which are coamplified (Elkahloun *et al.*, 1997). Only

functional analysis of the products of these genes will ultimately determine the significance of their amplification in tumors in which multiple copies of the 12q13.3 region is found. Therefore, the discovery of OS-9 as a binding partner of the oncoprotein Myc is intriguing in terms of the function of Myc and the potential role of OS-9 in human cancer.

Myc's ability to transform cells depends upon an intact amino terminal MHR II and the carboxyl terminal basic helix-loop-helix/leucine zipper. Specifically, deletion of MHR II from c-Myc results in a protein that is 2-fold more active than wild-type c-Myc in transcription assays but is functionally unable to transform cells (Brough *et al.*, 1995). Since OS-9 interacts with MHR II, the effect of OS-9 on Myc-mediated transformation was assessed. **Figure 5.1** shows that neither the cytoplasmically-localized OS-9(1-667) or the nuclear-localized OS-9(296-667) protein affects *H-ras*- or *H-ras* plus *v-myc*-induced cellular transformation. The focus formation experiments were performed in C3H10T1/2 cells which express OS-9 (Dorsey *et al.*, in prep). Perhaps the endogenous levels of OS-9 protein in these cells is already contributing to the number of foci formed in C3H10T1/2 cells, so exogenously added OS-9 is ineffective in these assays. On the other hand, perhaps OS-9 mediates functions of Myc that are peripheral to the process of cellular transformation.

The ability of both cytoplasmically-located and nuclear-localized OS-9 to inhibit Myc-mediated transcriptional activation of synthetic and natural reporter genes (**Figures 5.2-5.5**) demonstrates a potential role for the OS-9/Myc interaction. **Figure 5.2** illustrates that merely a two-fold molar excess of full-length OS-9 plasmid DNA over the GAL4-v-Myc(-32-219) activator results in greater than 70% reduction of transcriptional activation by Myc. A similar reduction in c-Myc transcriptional activity was only observed when a 20-fold molar excess of p107 was co-expressed with GAL4-c-Myc(1-210) (Gu *et al.*, 1994). Comparison of these results suggests the OS-9 effect on Myc transcriptional activation is quite strong and efficient, even at low concentrations. Also, the ability of both forms of OS-9 to repress Myc transactivation (**Figures 5.2 and 5.3**) suggests that OS-9 is not acting to repress Myc activity by sequestering it in the cytoplasm. It is theoretically possible that as the full-length OS-9 protein is retained in the cytoplasm of cells, it interacts with Myc and effectively concentrates the Myc protein in the cytoplasm of cells where it is unable to activate transcription (Ptashne and Gann, 1990; Martin, 1991). However, since the nuclear form of OS-9 (residues 296-667) is also an efficient repressor of Myc transactivation (**Figure 5.3**), this suggests cellular location is not a factor in these assays. It is possible that enough OS-9(1-667) leaks into the nucleus due to the level of overexpression in these transient assays, or perhaps the full-length OS-9 protein is modified in some way or

under a certain cellular condition that allows it to be translocated to the nucleus where it is then free to interact with Myc. Interestingly, a mutation in the nuclear localization region of the v-Myc protein (see **Figure 1.1**) that causes v-Myc to be retained in the cytoplasm does not affect the ability of v-Myc to transform fibroblasts (Min and Taparowsky, 1992; Tikhonenko *et al.*, 1993). This implies that production/retention of Myc exclusively in the nucleus of cells is not essential for all functions of Myc. The association and functional consequence of the interaction between Myc and a cytoplasmically-localized protein (OS-9) is therefore physiologically relevant.

The repression of both Myc and VP16 transactivation by OS-9 suggests that OS-9 may be a common factor mediating activation by these two distinct proteins. This supports previous studies from the laboratory in which Min *et al.* (1994) showed that overexpressing the VP16 TAD in the absence of a DNA binding domain could “squelch” transcriptional activation of a GAL4-v-Myc(-32-244) fusion protein bound to DNA. Additionally, both the v-Myc and VP16 TAD are able to reduce focus formation by pT24 *H-ras* and to inhibit the growth of colonies in which they are stably expressed. The transcription activation domain of MyoD was inefficient at inhibiting cellular transformation by H-Ras or “squelching” the transcriptional activity of either GAL4-v-Myc(-32-244) or GAL4-VP16(413-490) (Min *et al.*, 1994). The activity of the OS-9 protein supplements these observations since OS-9 can repress both v-Myc and VP16 transactivation but not MyoD-mediated transcriptional activation. While OS-9 does not affect cellular transformation by H-Ras or H-Ras and v-Myc, it could theoretically interact with a factor uniquely essential for transcriptional activation by these two proteins. Both the acidic activation domains of Myc and VP16 have been shown to interact specifically with the TATA binding protein TBP (Hateboer *et al.*, 1993; Stringer *et al.*, 1990). Whether the presence of OS-9 influences the Myc-TBP or VP16-TBP interaction and subsequent activation of transcription remains to be tested.

Testing the ability of OS-9 to repress transcriptional activation by full-length v-Myc was critical to demonstrate that the physiologically relevant form of Myc is influenced by OS-9 (**Figure 5.5**). In turn, these experiments demonstrated that it is a Myc/Max heterodimer bound to DNA that is recognized by OS-9. The (MBS)₃ TK CAT is under the transcriptional control of three tandem Myc E boxes, and it is the Myc/Max heterodimer that binds to and activates this gene from these consensus sites (Blackwell *et al.*, 1990). Hence, Myc is a target for OS-9 repression when participating in its functionally active complex with the cellular protein Max.

To begin to analyze the mechanism by which OS-9 represses Myc transactivation, the p(LexA)₈ (GAL4)₅ CAT reporter was utilized. This reporter was initially designed to test the repressor properties of the basic helix-loop-helix protein, Thing 1 (Hollenberg *et al.*, 1995). The p(LexA)₈ (GAL4)₅ CAT reporter discriminates between events that could be taking place and resulting in the repression of Myc and VP16 transactivation. For example, if the interaction between OS-9 and the v-Myc TAD occludes a site on v-Myc that is absolutely necessary for transcriptional activation, such as a site that would interact with the basal transcription machinery, then co-expression of GAL4-OS-9 in this system would still only affect Myc activation. However, if the interaction of OS-9 and v-Myc results in bringing OS-9 to the proximity of the promoter where OS-9 could then modulate transcriptional activation, then artificially binding OS-9 to DNA near the promoter of the reporter should result in the repression of every LexA activator examined.

Figure 5.6 depicts the activation properties of several LexA fusion proteins. By tethering the OS-9 protein to DNA via a heterologous DNA binding domain (GAL4), the transcriptional activity of all LexA fusion proteins is repressed. This implies that OS-9 does indeed function at the promoter, and it is the specific interaction with the v-Myc TAD that brings OS-9 to this region. Our data also suggest that VP16 is likely to directly bind OS-9 as well since the VP16 TAD is repressed by OS-9. However, there is no obvious sequence similarity between the TADs of v-Myc and VP16, and no *in vivo* or *in vitro* binding assays have been performed.

The ability of Myc to down-regulate certain cellular and viral promoters correlates with the presence of an initiator region, or Inr (Smale and Baltimore, 1989), within the Myc-targeted gene (Li *et al.*, 1994; Philipp *et al.*, 1994; Roy *et al.*, 1993; Du *et al.*, 1993). Repression by Myc of an Inr-containing promoter was first detected on the adenovirus-2 major late promoter. The enhancement of Myc-mediated repression on both the adenovirus-2 promoter and the cellular *adrenomedullin* promoter by OS-9 was surprising, since logically it was hoped that OS-9 would reverse Myc-mediated transcriptional repression (**Figures 5.8 and 5.9**). Another recently-identified Myc-interacting protein termed Miz-1 (for Myc-interacting zinc finger protein) also is involved in gene repression by Myc, but this protein interacts with the carboxy-terminus of Myc rather than the amino terminus (Peukert *et al.*, 1997). This at least suggests multiple pathways for the repression phenomenon. By making Myc a more efficient repressor, any “leaky” transcripts from a Myc down-regulated promoter could possibly be avoided. For example, **Figure 5.9** shows that v-Myc can suppress the AM-CAT reporter by approximately 70%. This means, however, that some *adrenomedullin* mRNA is still transcribed. In the presence of v-Myc and OS-9, only 5% residual activity of the AM-CAT reporter is observed. Interestingly, in cell lines transformed by various oncogenes (*v-myc*, *H-ras*, *E1A*), *adrenomedullin* gene expression is

very low (Wang *et al.*, submitted), suggesting a possible role for the AM protein in transformation. The amplification of OS-9 in several osteosarcomas implies it could have tumor-promoting properties. If overexpressed in the same cell type, OS-9 and Myc would virtually eliminate *adrenomedullin* expression, probably allowing the cell to become highly transformed and aggressive. This could be an example of the importance of the enhancement of repression of Myc by OS-9.

Finally, the ability of Myc to induce apoptosis seems to be counterintuitive to its role as an inducer of cellular proliferation and transformation. It has been suggested that Myc-mediated apoptosis is a “fail-safe” mechanism to remove cells which are inappropriately expressing *c-myc* or expressing mutant *c-myc* to avoid the progression of these cells to the malignant phenotype (reviewed in Packham and Cleveland, 1995). In any event, when cellular proliferation is blocked artificially and *c-myc* expression is induced, cells undergo apoptosis, and this event is dependent upon an intact MHR II (Evan *et al.*, 1992). To investigate whether OS-9 expression affects this function of Myc, OS-9-5 -a cell line stably expressing both v-Myc and OS-9(1-667) (**Figure 5.11**) -was generated. The biochemical event studied most thoroughly in apoptotic cells is the double-stranded cleavage of nuclear DNA in the linker regions between nucleosomes. This cleavage results in DNA fragment ladders representing multiples of 185 bp units that are easily identified on an agarose gels (Tomei and Cope, 1991). C3H10T1/2 cells, however, do not undergo the traditional double-stranded DNA cleavage in the internucleosomal linker region, so the identification of apoptotic C3H10T1/2 cells using this method is impossible (Tomei *et al.*, 1993). Since both Myc neo 13A and OS-9-5 cells are derived from C3H10T1/2 cells (see Materials and Methods), another detection method for apoptosis was utilized. Interestingly, when C3H10T1/2 cells become apoptotic, they detach from the plate and become suspended in the culture medium (Boyle *et al.*, 1997). Both the apoptotic (floating) and non-apoptotic (adherent) cells are collected, and fluorescence activated cell sorting (FACS) analysis is performed. End-labeled DNA due to pH-sensitive fragmentation during apoptosis of C3H10T1/2 cells is quantified using FACS. As shown in **Figure 5.13**, only 5% of the total amount of C3H10T1/2 cells (both floating and adherent) when starved are apoptotic compared to 30.8% of starved Myc neo 13A cells (**Figure 5.14**). This is the first direct measurement of apoptosis in the Myc neo 13A cell line. However, only 11.4% of all OS-9-5 cells when starved are apoptotic (**Figure 5.15**), indicating that the Myc-induced apoptosis in these cells is modified by the overexpression of OS-9. Interestingly, a transcriptional target of Myc, the *ornithine decarboxylase* gene (Bello-Fernandez *et al.*, 1993), has been implicated as both necessary for cellular proliferation and as a mediator of Myc-induced apoptosis (Packham and Cleveland,

1994). ODC catalyzes the rate-limiting step in polyamine synthesis (Tabor and Tabor, 1984), critical for cell division. However, polyamine synthesis is firmly regulated within the cell, and excess polyamines (that could result from the activation of *ODC* gene expression by Myc) are catabolized by various oxidases that generate reactive oxygen species (ROS; Sieler *et al.*, 1985). Normally cells are protected from the damaging effects of ROS by cellular detoxifying enzymes; however, sometimes the highly toxic hydroxyl radical is generated before these enzymes can act, and apoptosis is induced (reviewed in Packham and Cleveland, 1995). It is therefore possible that by decreasing the transactivation potential of Myc, OS-9 mediates decreased ODC activity which, in turn, results in less ROS production and reduced apoptotic activity. This could explain the differences in apoptosis noted in the various cell lines tested in this study.

Groups	Efficiency of Focus Formation
v-Myc	0% (1)
OS-9(1-667)	0% (1)
OS-9(296-667)	0% (1)
H-Ras	34% (8)
H-Ras + OS-9(1-667)	41% (4)
H-Ras + OS-9(296-667)	50% (3)
H-Ras + v-Myc	100% (8)
H-Ras + v-Myc + OS-9(1-667)	81% (3)
H-Ras + v-Myc + OS-9(296-667)	95% (2)

Figure 5.1 Transforming abilities of H-Ras and/or H-Ras plus v-Myc in the presence or absence of overexpressed OS-9 proteins. C3H10T1/2 cells were stably transfected with 200 ng of pT24 *H-ras*, 600 ng of pMC29 *v-myc*, and 2 μ g of *OS-9(1-667)* or *OS-9(296-667)* in the indicated combinations. After 14 days of growth in reduced serum medium, cells were fixed, stained, and examined for cellular transformation as described in Materials and Methods. The number of foci formed by the cooperation of H-Ras and v-Myc is set at 100%, and the level of focus formation obtained in other groups is expressed as a percentage of that value. The number in parentheses indicates the numbers of trials each experiment was performed.

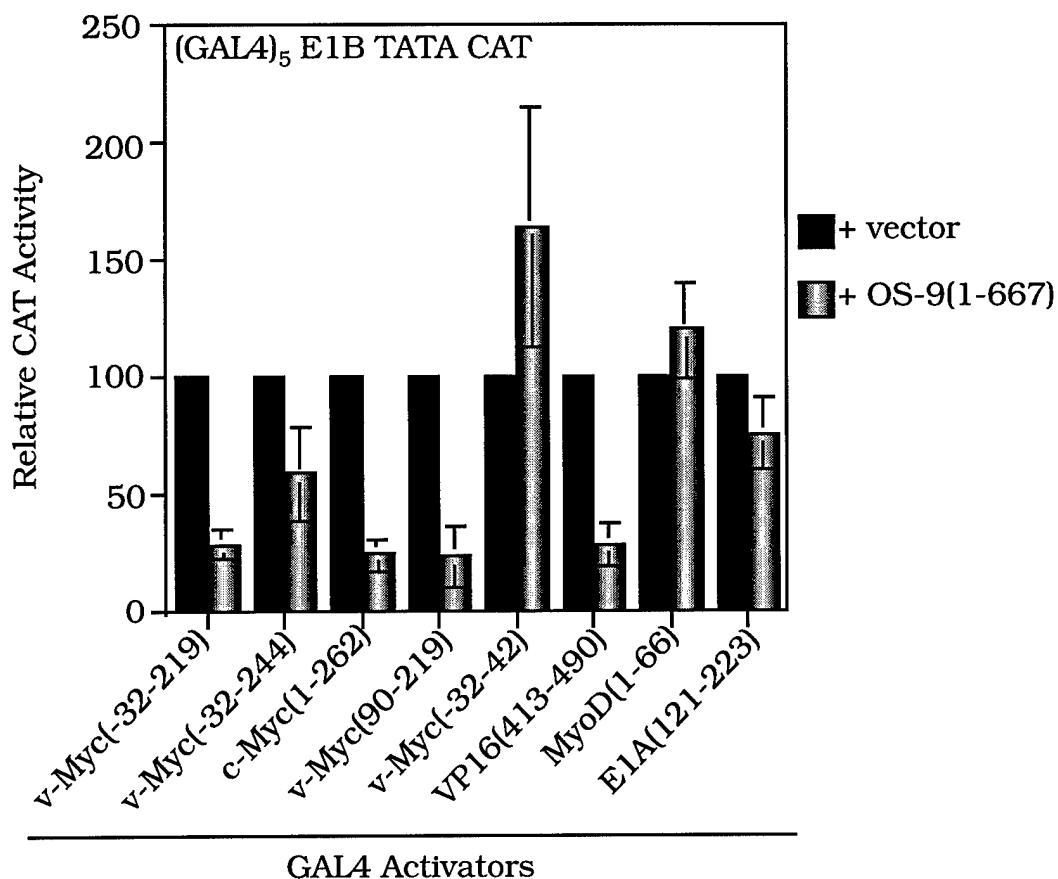


Figure 5.2 Transcriptional repression of certain GAL4 activators by overexpression of OS-9(1-667). C3H10T1/2 cells were transiently transfected with 5 μ g of each GAL4 activator, 5 μ g of the (GAL4)₅ E1B TATA CAT reporter, and a 2-fold molar excess of full-length OS-9(1-667) plasmid DNA. CAT activity was determined as outlined in Materials and Methods. The CAT activity of each GAL4 activator in the presence of control vector is set at 100% and shown in the black bars, and the residual CAT activity of the GAL4 activators when OS-9 is co-expressed is expressed as a percentage of that value, represented in the shaded bars in the graph. Each experiment was performed independently a minimum of three times.

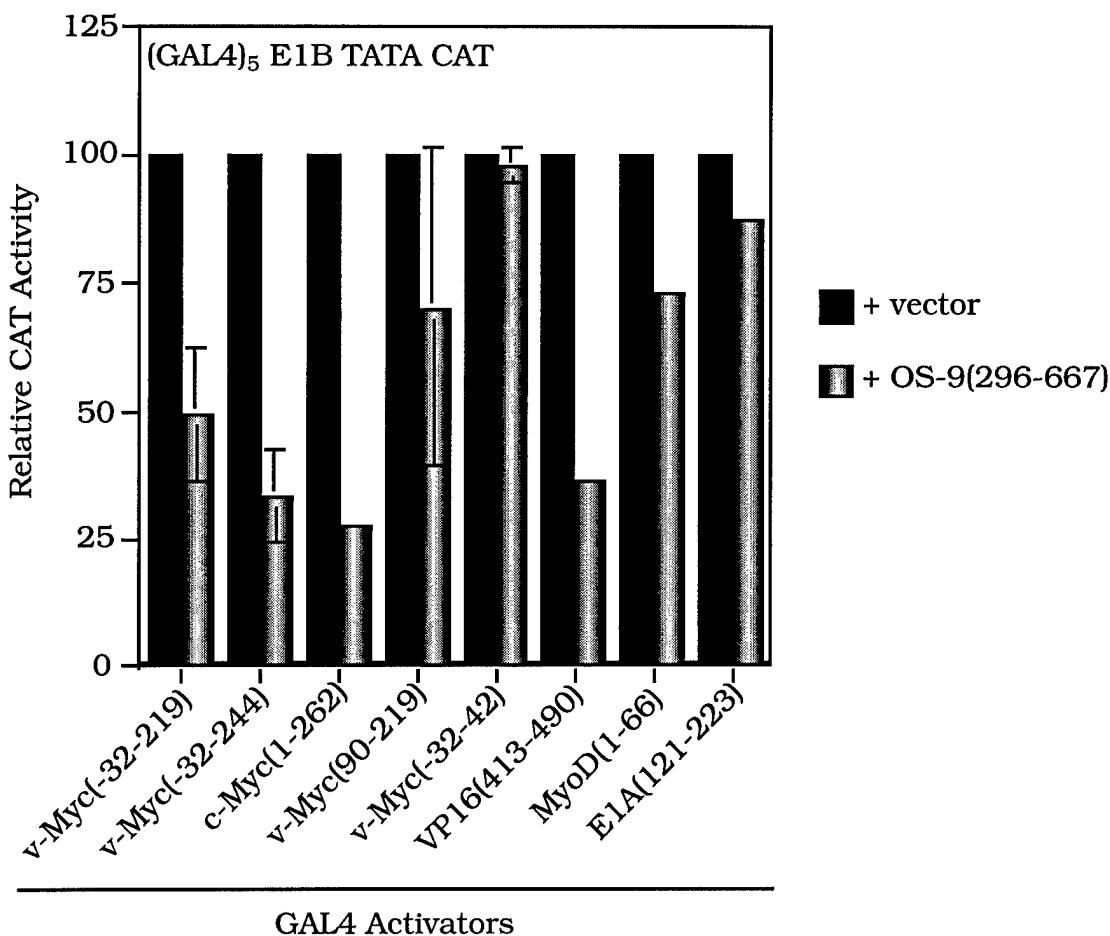


Figure 5.3 Transcriptional repression of GAL4 activators by overexpression of OS-9(296-667). C3H10T1/2 cells were transiently transfected with 5 μ g of each GAL4 activator, 5 μ g of the (GAL4)₅ E1B TATA CAT reporter, and a 2-fold molar excess of OS-9(296-667) plasmid DNA. CAT activity was determined as described in Materials and Methods. The CAT activity of each GAL4 activator in the presence of control vector is set at 100% and is shown by the black bars; the residual CAT activity of GAL4 activators in the presence of OS-9 is represented as a percentage of that value and is depicted by the shaded bars. Each experiment was performed independently a minimum of two times, and the groups with no error bars had little variation between experiments.

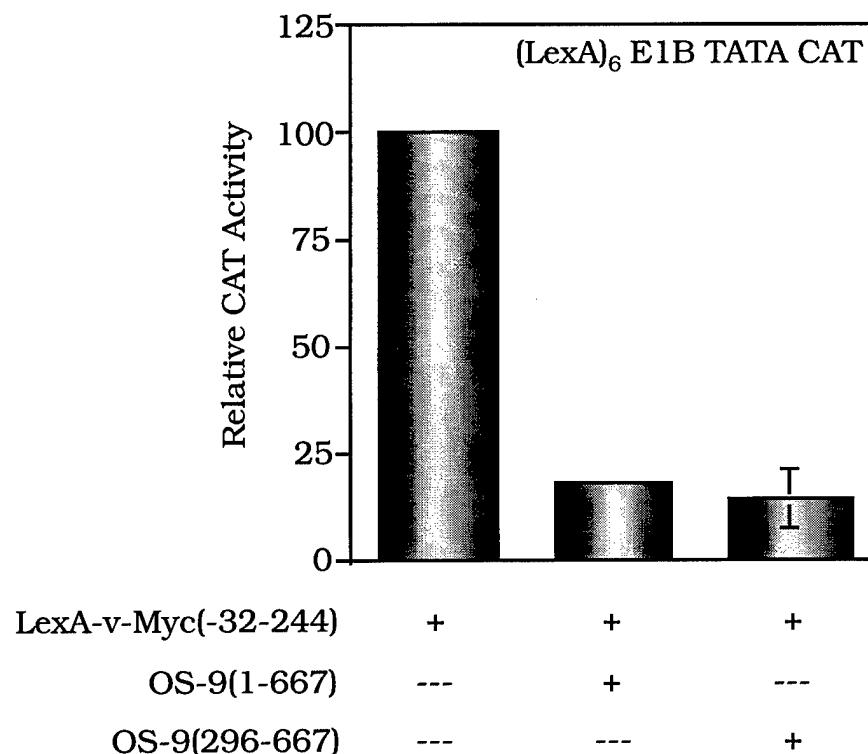


Figure 5.4 Inhibition of the LexA-v-Myc(-32-244) activator by OS-9. C3H10T1/2 cells were transiently transfected with 5 μ g of LexA-v-Myc(-32-244), 5 μ g of the (LexA)₆ E1B TATA CAT reporter, and a 2-fold molar excess of either OS-9(1-667) or OS-9(296-667) plasmid DNA. CAT activity was determined as described in Materials and Methods. The CAT activity generated by LexA-v-Myc(-32-244) alone is set at 100%, and the resulting CAT activity in cells overexpressing the OS-9 proteins is represented as a percentage of that value. Error bars represent the variation between experiments. No measurable variation was observed in the samples in which LexA-v-Myc(-32-244) and OS-9(1-667) were co-expressed.

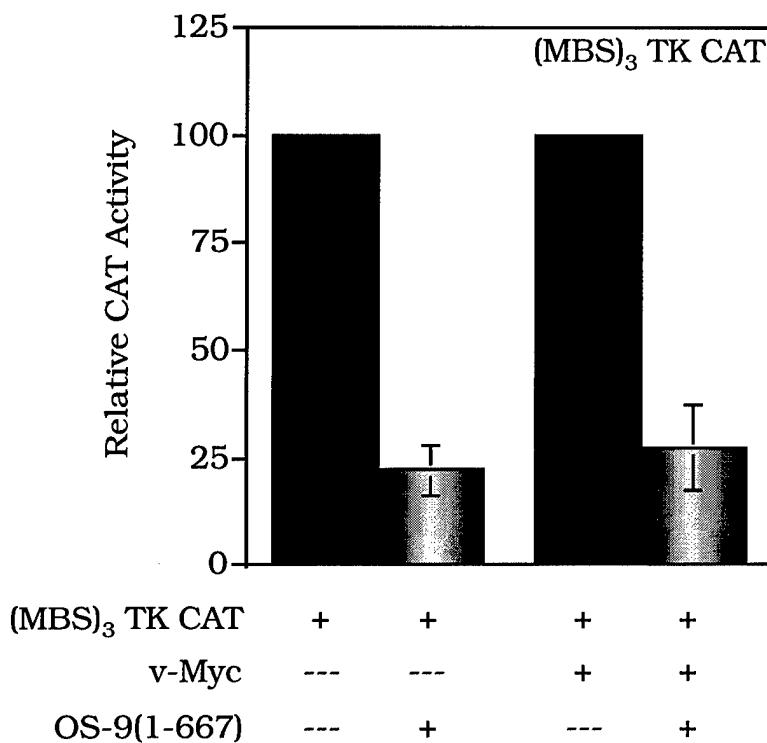


Figure 5.5 Transcriptional repression of wild-type v-Myc and the Myc-responsive reporter (MBS)₃ TK CAT. C3H10T1/2 cells were transiently transfected with 2 μ g of (MBS)₃ TK CAT, 5 μ g pMC29 *v-myc*, and 10 μ g *OS-9(1-667)*. CAT activity was determined as described in Materials and Methods. The CAT activity of either the reporter alone or the reporter plus v-Myc (black bars) is set at 100%, and the residual CAT activity observed following co-expression of v-Myc and OS-9(1-667) is represented as a percentage of that value (shaded bars). Error bars represent the variation between experiments. Each experiment was performed independently a minimum of three times.

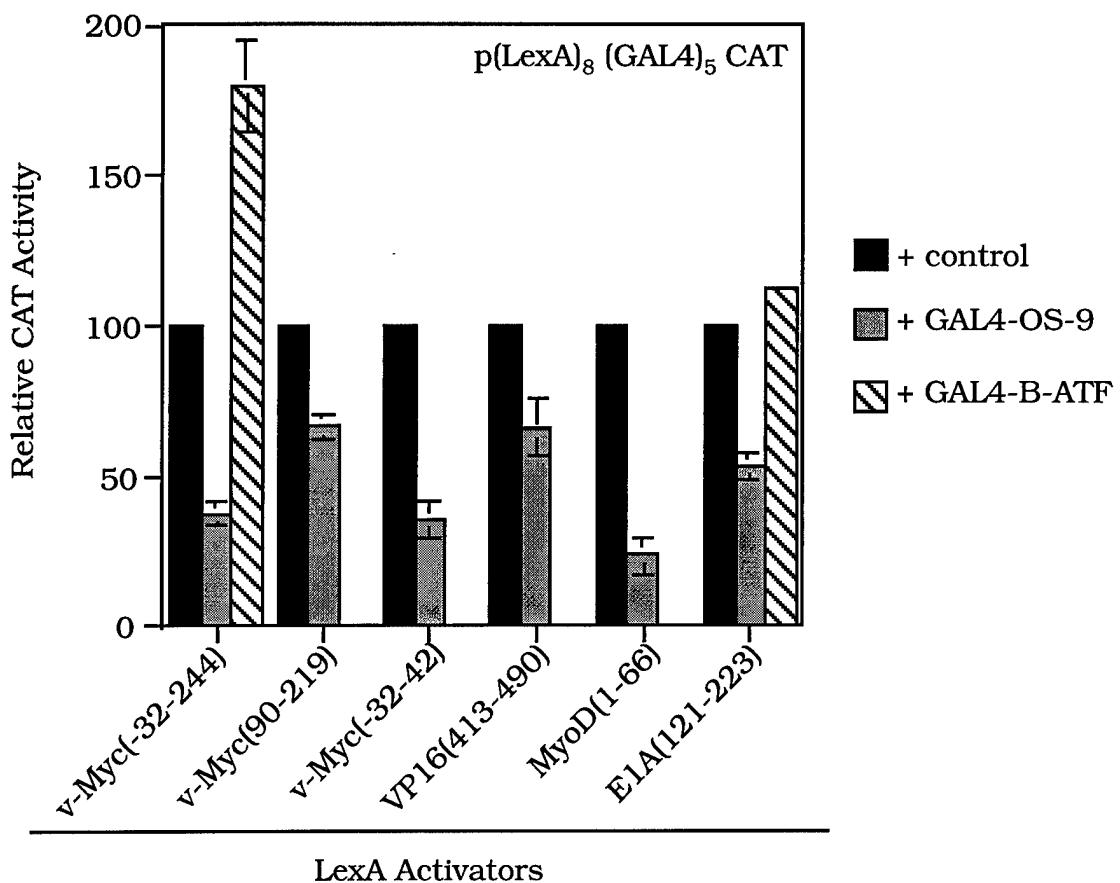


Figure 5.6 Transcriptional repression of LexA activators by DNA-bound GAL4-OS-9(120-667). C3H10T1/2 cells were transiently transfected with 5 μ g of the complex reporter p(LexA)₈ (GAL4)₅ CAT, 1 μ g of each LexA activator, and 5 μ g of each GAL4 fusion. CAT activity was determined as outlined in Materials and Methods. The CAT activity of each LexA activator expressed alone is set at 100% (black bars), and the residual CAT activity of the LexA activators following co-expression of GAL4-OS-9 or GAL4-B-ATF is represented as a percentage of that value (gray bars and striped bars, respectively). The error bars represent the variation obtained between experiments. Each experiment was performed independently a minimum of three times.

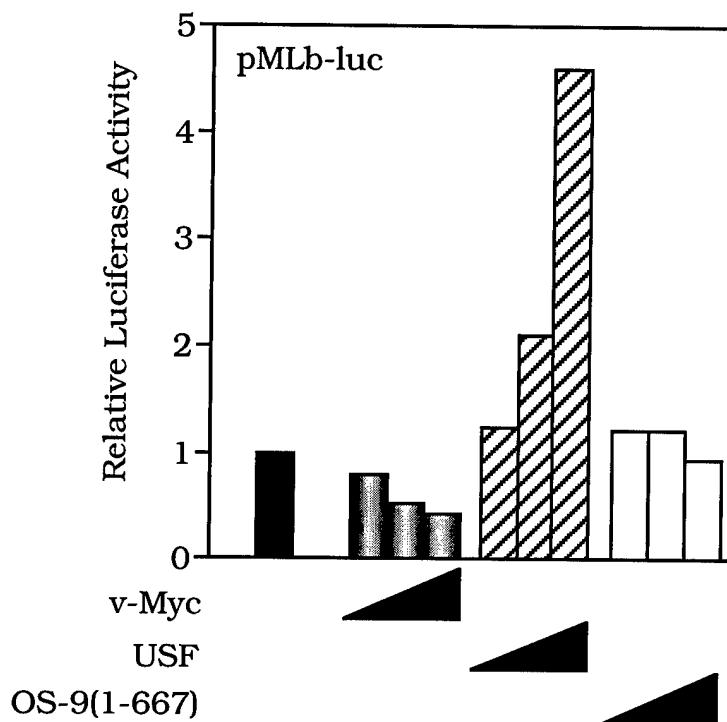


Figure 5.7 Regulators of the adenovirus-2 major late promoter. C3H10T1/2 cells were transiently transfected with 5 µg of the pMLb-luc reporter and 2, 5, and 10 µg of pMC29 *v-myc*, USF, or OS-9(1-667). Luciferase activity was determined as described in Materials and Methods. The basal level of luciferase activity of the pMLb-luc reporter is set at 1.0 (black bar), and the activities in the presence of increasing amounts of v-Myc (shaded bars), USF (striped bars), or OS-9(1-667) (white bars) are expressed as a fold increase or decrease of that value. Each experiment was performed independently at least twice, and no measurable variation was observed.

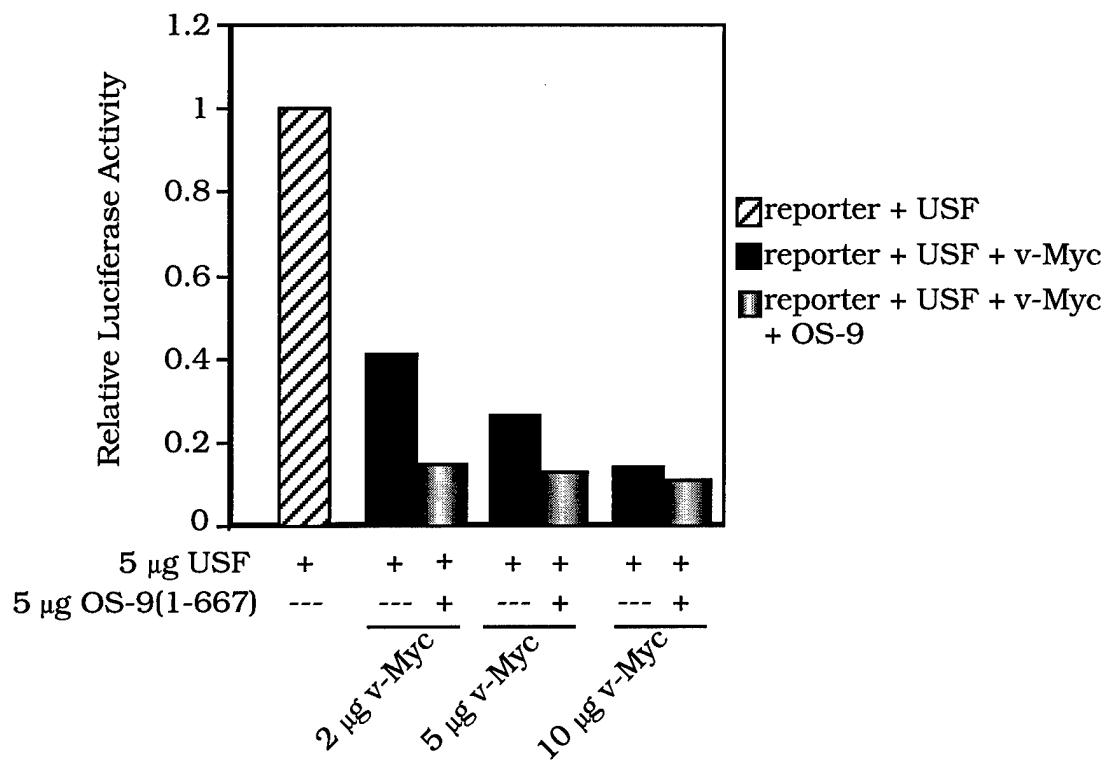


Figure 5.8 Enhanced transcriptional repression by v-Myc due to overexpression of OS-9(1-667). C3H10T1/2 cells were transiently transfected with 5 μ g of the pMLb-luc reporter, 5 μ g of USF, and either 2, 5, and 10 μ g of pMC29 *v-myc* plus (+) or minus (---) 5 μ g of *OS-9(1-667)*. Luciferase activity was determined as described in Materials and Methods. The activity obtained from the reporter and 5 μ g of USF is set at 1.0 (striped bar), and the activity of the promoter when increasing amounts of v-Myc (black bars) or v-Myc plus OS-9(1-667) (shaded bars) is represented as a percentage of that value. Each experiment was performed in duplicate a minimum of two times, and no significant error was observed between experiments.

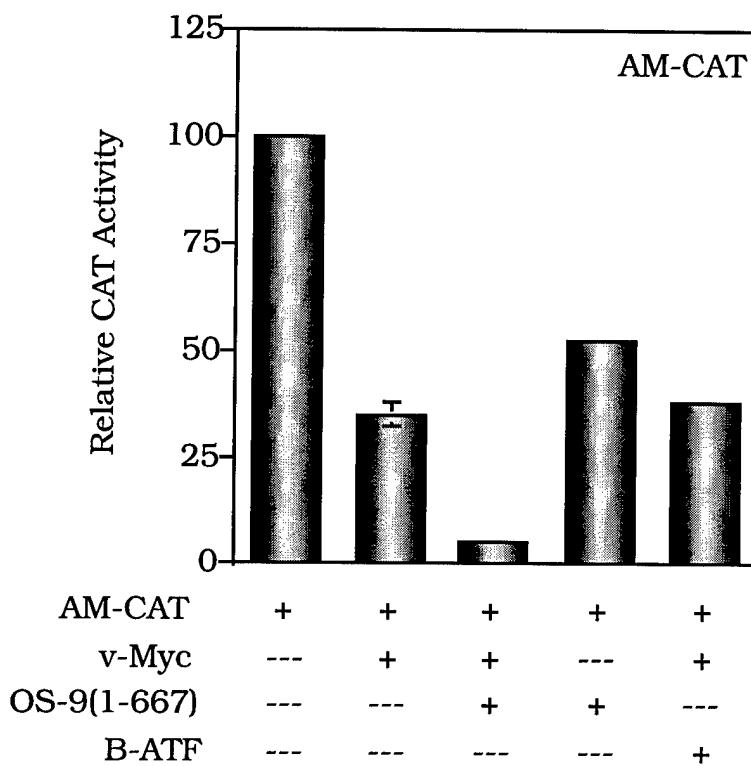


Figure 5.9 Enhanced transcriptional repression of the AM-CAT reporter by v-Myc due to overexpression of OS-9(1-667). C3H10T1/2 cells were transiently transfected with 2.5 μ g of the AM-CAT reporter, 5 μ g of pMC29 *v-myc*, and 10 μ g of *OS-9(1-667)* or *B-ATF(1-125)*. CAT activity was determined as described in Materials and Methods. The activity of the AM-CAT reporter alone is set at 100%, and the resulting activity following co-expression of the indicated proteins is represented as a percentage of that value. Each value is the result of four independent experiments, and no measurable error was obtained between experiments, except for the sample in which the error bar is indicated.

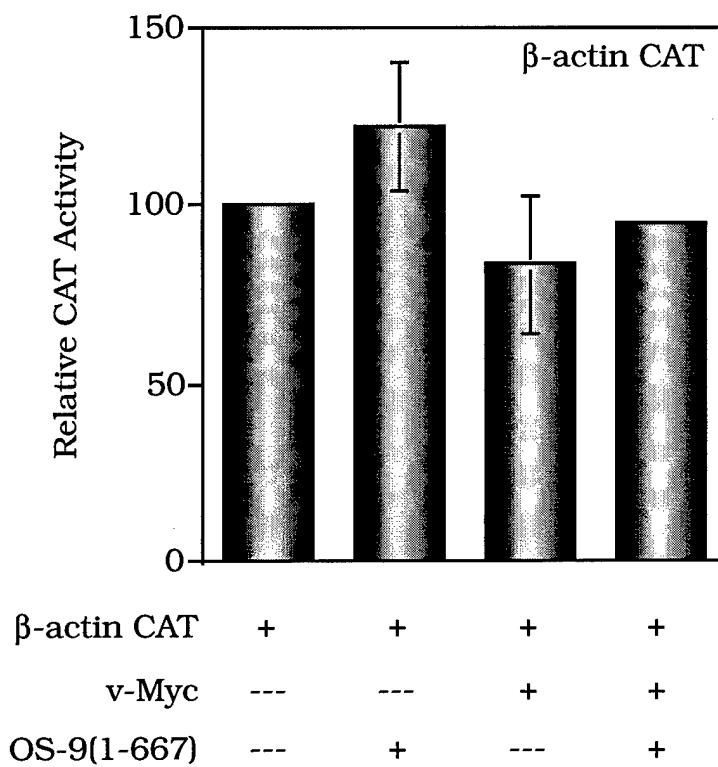


Figure 5.10 OS-9(1-667) does not negatively influence the expression of β -actin CAT, a non-Myc regulated reporter gene. C3H10T1/2 cells were transiently transfected with 2.5 μ g of the β -actin CAT reporter, 5 μ g of pMC29 *v-myc*, and 10 μ g of *OS-9(1-667)*. CAT activity was determined as described in Materials and Methods. The level of activity of β -actin CAT is set at 100%, and the level in the presence of v-Myc, OS-9, or v-Myc plus OS-9 is expressed relative to that amount. Error bars represent the variation observed between experiments. Each value is the result of three independent experiments.

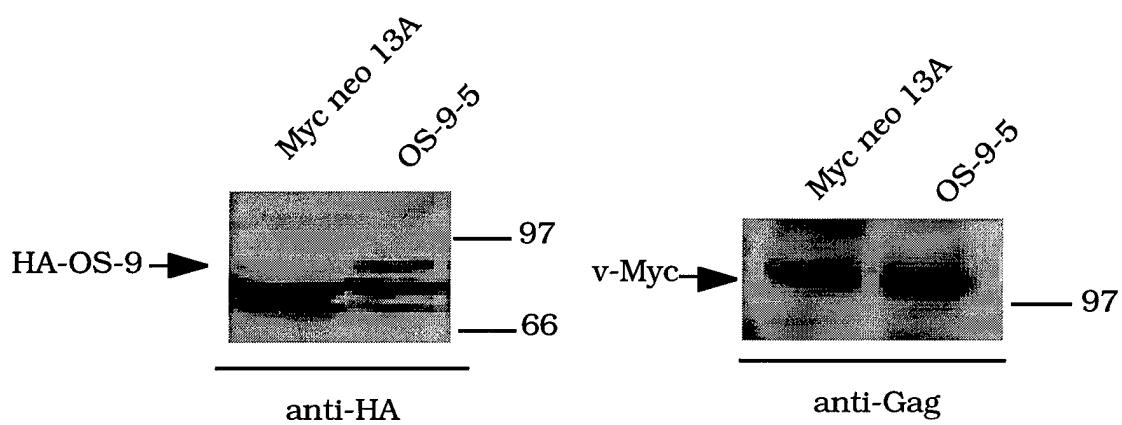


Figure 5.11 Protein expression in Myc neo 13A and OS-9-5 cells. Whole cell extracts of subconfluent Myc neo 13A cells and OS-9-5 cells were prepared, separated by SDS-PAGE, transferred to nitrocellulose, and incubated with an anti-HA antibody (left panel) to detect OS-9 expression and with an anti-Gag antibody (right panel) to detect v-Myc expression (see Materials and Methods for details). Molecular weight standards are indicated to the right of each blot, and arrows designate the migration of the proteins of interest.

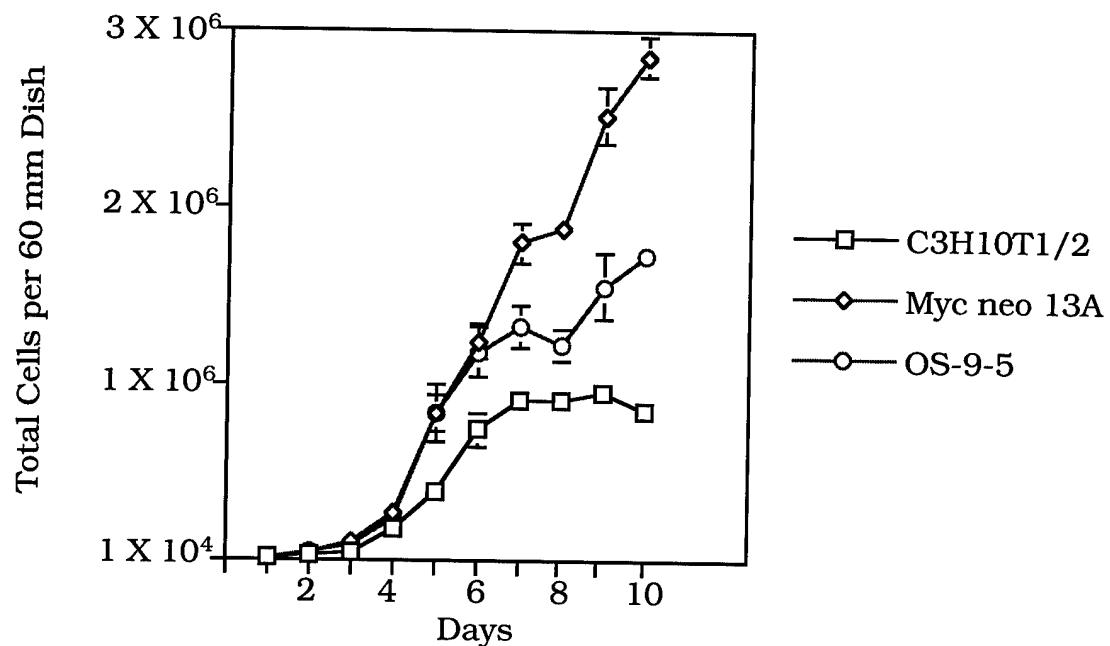


Figure 5.12 Growth properties of three related C3H10T1/2 cell lines. The parental C3H10T1/2 fibroblasts, Myc neo 13A cells, and OS-9-5 cells [overexpressing both v-Myc and OS-9(1-667)] were seeded at 1×10^4 cells per 60 mm dish in growth medium (BME + 10% FBS + P/S). Medium was replaced every three days, and the total cell number per plate was determined on 9 consecutive days for each cell type. The graph shows the total number of cells counted at each time point for the duration of the counting period.

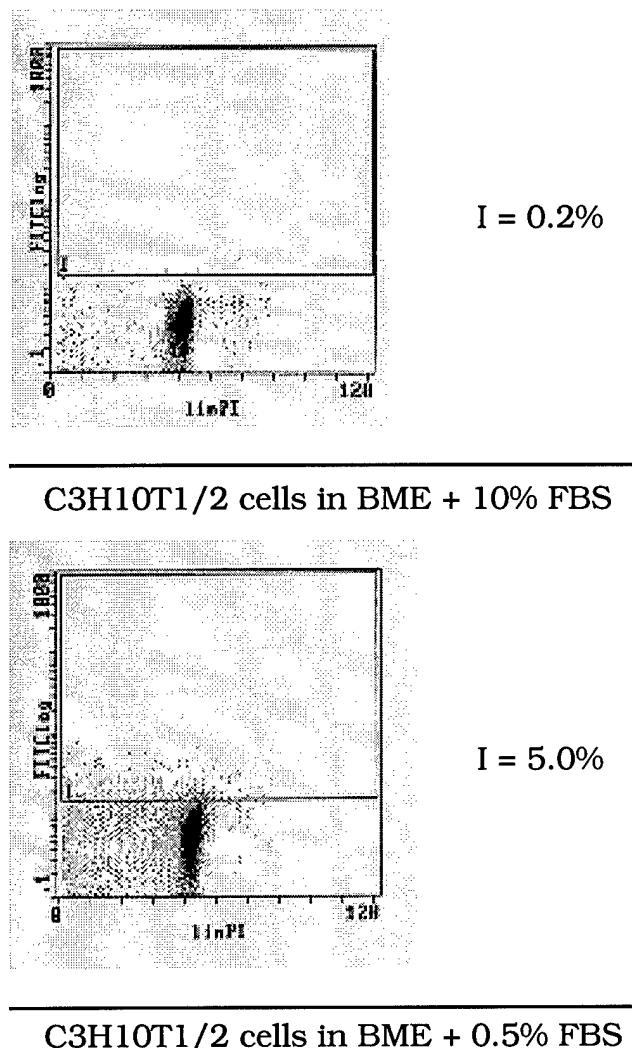


Figure 5.13 Analysis of apoptosis in C3H10T1/2 cells. C3H10T1/2 cells were grown to subconfluence in growth medium (BME + 10%FBS + P/S; top panel) or allowed to reach subconfluence and then maintained in starvation medium (BME + 0.5% FBS + P/S; bottom panel) for 48 hours. Suspended and adherent cells were collected, prepared, and subjected to analysis by fluorescence activated cell sorting (FACS) as described in Materials and Methods. The percentage of cells undergoing apoptosis was established for both growth conditions. "I" indicates the apoptotic cells in each sample.

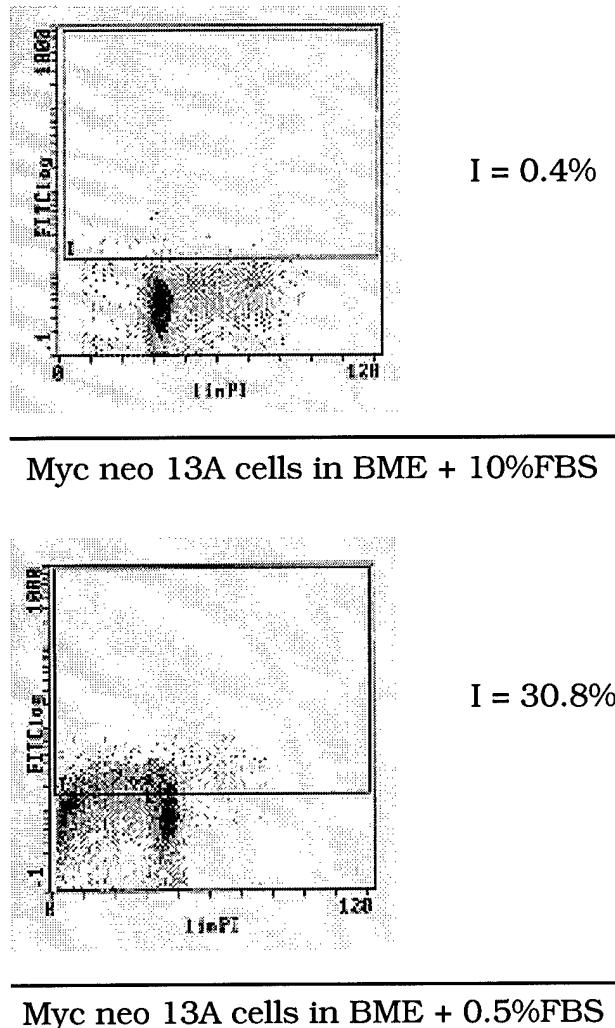


Figure 5.14 Analysis of apoptosis in Myc neo 13A cells. Myc neo 13A cells were grown to subconfluence in growth medium (BME + 10%FBS + P/S; top panel) or allowed to reach subconfluence and then maintained in starvation medium (BME + 0.5% FBS + P/S; bottom panel) for 48 hours. Suspended and adherent cells were collected, prepared, and subjected to analysis by fluorescence activated cell sorting (FACS) as described in Materials and Methods. The percentage of cells undergoing apoptosis was established for both growth conditions. “I” indicates the apoptotic cells in each sample.

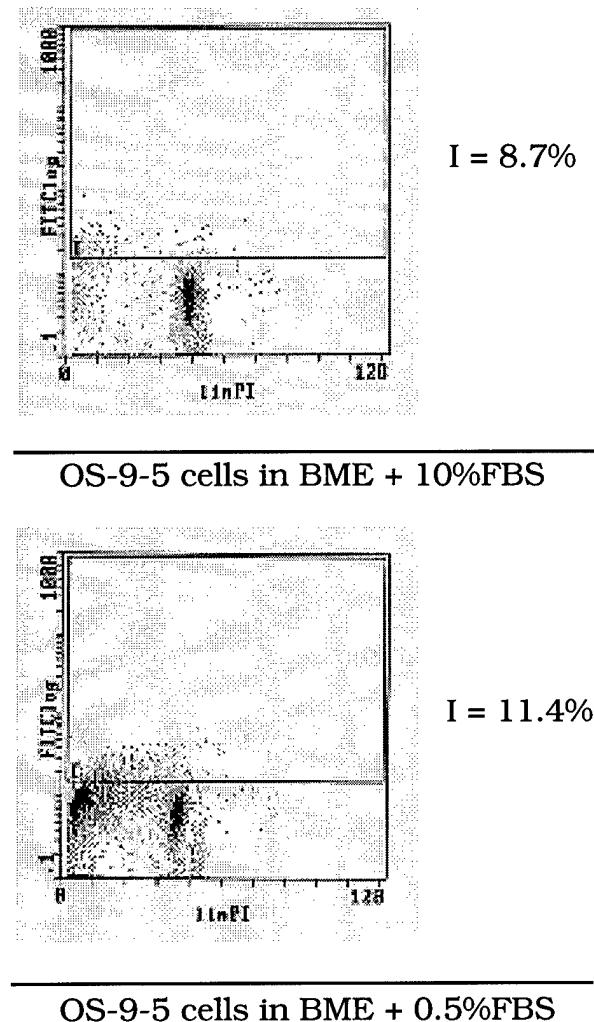


Figure 5.15 Analysis of apoptosis in OS-9-5 cells. OS-9-5 cells were grown to subconfluence in growth medium (BME + 10%FBS + P/S; top panel) or allowed to reach subconfluence and then maintained in starvation medium (BME + 0.5% FBS + P/S; bottom panel) for 48 hours. Suspended and adherent cells were collected, prepared, and subjected to analysis by fluorescence activated cell sorting (FACS) as described in Materials and Methods. The percentage of cells undergoing apoptosis was established for both growth conditions. “I” indicates the apoptotic cells in each sample.

CHAPTER 6: FACTORS THAT OPPOSE MYC FUNCTION IN RAS-MEDIATED CELLULAR TRANSFORMATION

Introduction

AP-1 (activating protein-1) proteins represent a family of transcription factors composed of dimers between Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra1, and Fra2), or ATF (activating transcription factor; ATF2, ATF3/LRF1, and B-ATF) proteins (Vogt and Bos, 1990; Angel and Karin, 1991). The AP-1 components interact via a leucine zipper motif and bind DNA through a stretch of basic amino acids (Kouzarides and Ziff, 1988). The AP-1 transcription factor complex is involved in the regulation of cellular proliferation and cell survival (Karin *et al.*, 1997). In 1995, a cDNA from a human B cell library encoding a 125 amino acid nuclear-localized protein containing a basic leucine zipper was isolated in our laboratory (Dorsey *et al.*, 1995). This protein, called B-ATF, shares extensive homology with the AP-1/ATF superfamily of bZIP transcription factors. B-ATF exhibits a tissue-restricted pattern, with expression noted in lung (Dorsey *et al.*, 1995), various EBV-positive B cell lymphoma cell lines (Dorsey *et al.*, 1995), and in human T lymphocyte cell lines such as Jurkat and Sup-T-1 (Johansen *et al.*, in prep; Hasegawa *et al.*, 1996). B-ATF interacts specifically with all Jun family members in GST pull-down assays but not with Fos (Dorsey *et al.*, 1995). Mammalian two-hybrid assays indicate that B-ATF interacts with Jun via the B-ATF leucine zipper and that these Jun/B-ATF heterodimers prefer to bind to TRE (TPA responsive elements) sites in DNA versus CRE (cAMP responsive elements) sites (Echlin *et al.*, in prep). Overexpression of B-ATF inhibits transcriptional activation by AP-1 in a dose-dependent manner, suggesting that B-ATF functions as a naturally-occurring, tissue-specific dominant negative to AP-1 function. Further analysis of the effect of B-ATF on cell growth and transformation was examined.

Besides natural dominant negatives like B-ATF, engineered dominant negative proteins have been created to analyze the functions of various transcription factors. A Jun protein deleted for its transcription activation domain proves to be an efficient repressor of cellular transformation (Lloyd *et al.*, 1991). Likewise, a Fos protein in which the TAD has been removed is an equally potent inhibitor of AP-1 activity (Brown *et al.*, 1994). In addition to this method of creating dominant negatives, removal of the basic DNA binding region from a transcription factor effectively abolishes activity of the wild type factors (MacGregor *et al.*, 1996; Kim and Spiegelman, 1996). In order for these types of dominant negatives to function as desired,

however, they must be overexpressed *in vivo*. This could lead to nonspecific events that interfere with data interpretation.

To avoid these complications, a novel approach to design dominant negatives applicable to many different transcription factors has been developed in which the basic DNA binding domain of these transcription factors is replaced by an acidic stretch of amino acids. This acidic region on the modified protein interacts with the basic region of the wild-type dimerization partner, and this heterodimer now forms a tight coiled-coil which stabilizes the complex. DNA binding is abolished at equimolar concentrations, thereby avoiding the need to vastly overexpress the dominant negative proteins to see physiological effects (Krylov *et al.*, 1995). This technology has been applied to the Fos and to the Max transcription factors. In the case of Fos, circular dichroism spectroscopy indicates that a mixture of Jun and modified acidic Fos protein (A-Fos) is 3000-fold more stable than a mixture of Jun and Fos protein in which the basic region of Fos is deleted (Olive *et al.*, 1997). The binding of AP-1 to DNA containing the consensus TRE site is completely inhibited by an equimolar concentration of A-Fos, and this inhibition is specific to AP-1 since a non-related transcription factor that was similarly modified does not affect DNA binding by AP-1 (Olive *et al.*, 1997). Finally, expression of an equal amount of Jun and A-Fos drastically reduces transcription activation by Jun on an AP-1-responsive reporter. Once again, this repression is specific since non-related modified acidic transcription factors do not affect the function of the AP-1 complex (Olive *et al.*, 1997). In the case of Max, the basic region of this helix-loop-helix/leucine zipper protein was also replaced by a novel acidic appendage to form A-Max-C (**Figure 6.6**; Krylov *et al.*, 1997). As was observed with A-Fos, A-Max-C, at an equimolar concentration, efficiently inhibits DNA binding of Myc and Max to E box-containing DNA (Krylov *et al.*, 1997). To further examine the roles of these dominant negatives, the effect on cellular transformation was assessed.

Results

B-ATF does not contain a transcription activation domain

The identification of a basic leucine zipper domain in the nuclear-localized B-ATF protein suggests this protein may function as a transcription factor *in vivo*. Additionally, the interaction of B-ATF with Jun family members and the ability of these complexes to bind AP-1 DNA further implies that B-ATF will impact AP-1-mediated activities within a cell (Dorsey *et al.*, 1995). To investigate whether B-ATF contains a TAD, the full-length B-ATF protein (residues 1-125), as well as two truncations of B-ATF (residues 1-32 and 81-125), were fused to the DNA

binding domain of the yeast activator protein GAL4. The ability of these fusions to activate transcription of the (GAL4)₅ E1B TATA CAT reporter was examined. C3H10T1/2 cells were transiently transfected with 5 µg reporter, 5 µg of each GAL4 fusion protein, and 5 µg of RSV-LacZ to control for transfection efficiency. **Figure 6.1** illustrates the results of these experiments. The GAL4(1-147) vector was used as a negative control, and the activity of GAL4 E1A was the positive activator in this study. As can be seen in **Figure 6.1**, neither the full-length (1-125) nor the truncated versions (1-32 and 81-125) of B-ATF are able to activate transcription from the (GAL4)₅ E1B TATA CAT reporter. This suggests that despite its ability to interact with Jun protein and subsequently bind as a complex to DNA, B-ATF does not provide sequences that function to activate transcription *in vivo*.

Overexpression of B-ATF modulates the growth properties of cells

Transient transfection experiments have shown that B-ATF can inhibit the transcriptional activity of AP-1 in a dose-dependent manner, suggesting that B-ATF behaves as a dominant negative to AP-1 (Echlin *et al.*, in prep). To examine the effect of B-ATF on the growth properties of cells, a C3H10T1/2 cell line was generated that overexpresses a full-length, HA-tagged B-ATF. B-ATF expression in the C3H10T1/2 B-ATF cell line (pDCR-B-ATF) was confirmed by Western blot analysis (Echlin *et al.*, in prep) using an antiserum against HA. The growth properties of this cell line were studied and compared to those of the C3H10T1/2 parental cell line and a C3H10T1/2-derived cell line that overexpresses the human H-Ras oncogene called Ras neo 11A (Weyman *et al.*, 1988). Growth curves were performed as outlined in Materials and Methods, and the results are plotted in **Figure 6.2**. C3H10T1/2 cell growth is represented by squares, and these cells reach a saturation density of approximately 1 X 10⁶ cells. Ras neo 11A cells (represented by diamonds) continue to grow until Day 9 of the counting period and reach a density of 2.75 X 10⁶ cells per plate. This continued growth reflects their highly transformed phenotype. Interestingly, the C3H10T1/2 B-ATF cell line (pDCR-B-ATF; represented by circles) grows much more slowly than either the C3H10T1/2 cells or Ras neo 11A cells, and the cells which overexpress B-ATF never reach as high a final cell number as either of the other cell lines at any time during the counting period (**Figure 6.2**). To examine if this phenomenon reflected slower growth, or just a toxic effect of B-ATF, C3H10T1/2 cells were stably transfected with either pDCR or pDCR-B-ATF (both of which can be selected for neomycin resistance) as described in Materials and Methods. Colonies were selected and counted. Overall, the same number of colonies were obtained from each experimental group (Echlin *et al.*, in prep), indicating that B-ATF is not toxic to cellular growth. However, as can be seen in the pictures in **Figure 6.3**, representative control and B-ATF-expressing colonies are

morphologically distinct. While the pDCR colonies form compact, organized units, the pDCR-B-ATF colonies are diffuse and less organized. The impression is that pDCR-B-ATF colonies are composed of fewer cells than the pDCR colonies, reflecting the influence of B-ATF on the rate of cell growth rather than cell viability.

B-ATF influences cellular transformation by H-Ras

Since it has been demonstrated that c-Jun activity is necessary for Ras-mediated cellular transformation (Johnson *et al.*, 1996), and because B-ATF has been shown to modulate AP-1 transcriptional activity (Echlin *et al.*, in prep), the effect of B-ATF on cellular transformation was analyzed. C3H10T1/2 cells (**Figure 6.4**) and NIH 3T3 cells (**Figure 6.5**) were stably transfected with 200 ng pT24 *H-ras* with or without 600 ng of the various *B-ATF* plasmids. **Figure 6.4** shows that the level of transformation achieved by H-Ras alone in C3H10T1/2 cells is set at 100%. Overexpression of the pDCR vector or pNLVP16 vector (which encodes the VP16 activation domain) does not affect Ras-mediated transformation; however, pDCR-B-ATF reduces the level of focus formation obtained with H-Ras by approximately 50%. Interestingly, expression of a fusion protein composed of B-ATF and the VP16 TAD increases cellular transformation by nearly 2.4-fold (**Figure 6.4**). This implies that B-ATF is indeed heterodimerizing with Jun and binding consensus AP-1 DNA, and the presence of the VP16 TAD on B-ATF now makes this complex transcriptionally active and a potent enhancer of cellular transformation. Neither the empty vectors nor the B-ATF proteins alone affect cellular transformation. **Figure 6.5** indicates these results are similar in NIH 3T3 cells as well. Expression of H-Ras induces transformation, and co-expression of B-ATF inhibits focus formation by 36%. While not as potent an inhibitor in NIH 3T3 cells as C3H10T1/2 cells, the trend in reduction of focus formation is similar between the two cell types. This indicates that B-ATF is able to function as a specific inhibitor of AP-1 activity in complex cellular assays such as focus formation.

Engineered dominant negatives of Fos and Max are potent inhibitors of cellular transformation

In an effort to fully assess the role of certain basic leucine zipper or basic helix-loop-helix/leucine zipper transcription factors *in vivo*, a collaboration with Dr. Charles Vinson from the Laboratory of Biochemistry at the National Cancer Institute, National Institutes of Health in Bethesda, Maryland was undertaken. In these studies, the basic regions of the c-Fos and Max proteins have each been removed and replaced with different stretches of acidic amino acids (**Figure 6.6**; Olive *et al.*, 1997; Krylov *et al.*, 1997). To determine the effect of overexpression of these proteins on cellular transformation and growth, C3H10T1/2 cells were stably transfected

with H-Ras and the different Fos proteins (**Figure 6.7**). B-Fos is a Fos protein that contains only the basic leucine zipper, 0-Fos represents only the leucine zipper of Fos, and A-Fos is the Fos leucine zipper appended to the acidic stretch of residues (**Figure 6.6**: Olive *et al.*, 1997). Additionally, another basic leucine zipper protein CREB (cAMP response element binding protein) was modified such that the basic region of CREB was replaced by an acidic sequence and used as a control in these experiments. As can be seen in **Figure 6.7**, a three-fold excess of B-Fos to H-Ras results in a reduction of focus formation of greater than 40%, presumably due to the formation of Jun/B-Fos heterodimers that can still bind to DNA but contain truncated, and thus inactive, Fos protein. Co-expression of 0-Fos further enhances inhibition of transformation by H-Ras. In this case, however, 0-Fos can still interact with Jun through its leucine zipper, but this complex is unable to bind to DNA since the Fos basic domain is missing. A-Fos is the most potent inhibitor of H-Ras-mediated transformation, suppressing focus formation by 70%. In this case, heterodimers between Jun and A-Fos are more thermodynamically stable than those formed between Jun and 0-Fos (Olive *et al.*, 1997). These complexes are also unable to bind DNA, and the result is effective inhibition of cellular transformation by H-Ras. As a control, CREB, a transcription factor involved in transcriptional activation of promoters containing cAMP response elements, was also modified by the removal of its basic DNA binding domain and replacement with an acidic appendage. As can be seen in **Figure 6.7**, this protein is not as efficient at inhibiting Ras focus formation as the Fos proteins. This indicates the specificity of the role of Fos (and AP-1 in general) in cellular transformation by Ras, and implies that these dominant negative proteins can function in complex biological assays.

To ensure the reduction in cellular transformation observed in **Figure 6.7** is not the result of a loss of cell viability, colony assays were performed to examine the effect of the dominant negatives on cellular growth. In this experiment, 1/6 of the cell suspension used to analyze focus formation in **Figure 6.7** was further diluted 1:6 and maintained in selection medium (BME + 10%FBS + 400 µg/ml G418) for two weeks. The number of colonies obtained with H-Ras alone was considered the positive control (**Figure 6.8**), and the number of colonies obtained in the other groups is expressed as a percentage of that value. While reduced colony formation is seen in each group co-expressing a modified Fos protein, the extent of inhibition of cell growth is much less than the inhibition of transformation. This implies that the impact of Fos dominant negative overexpression is important to cellular transformation but not to growth of C3H10T1/2 cells.

The role of v-Myc in enhancing cellular transformation by activated H-Ras is known and has been examined extensively in our laboratory (Min and Taparowsky, 1992; Min *et al.*, 1993). To examine how cellular transformation by Ras and Myc is affected by the basic helix-loop-helix/leucine zipper protein Max when its DNA binding domain is replaced by an acidic appendage, stable transfection of C3H10T1/2 cells was performed as described in Materials and Methods. **Figure 6.9** shows the results of these experiments. wt Max is the full-length Max protein, including the nuclear localization signal. D-Max-C contains the Max helix-loop-helix/leucine zipper without the basic domain of Max but including the carboxy terminus of Max which provides the nuclear localization signal. A-Max-C also contains the helix-loop-helix/leucine zipper and nuclear localization signal of Max, but the basic domain has been replaced by the acidic appendage (**Figure 6.6**; Krylov *et al.*, 1997). Mitf is the mouse microphthalmia protein, a helix-loop-helix/leucine zipper transcription factor involved in coat pigmentation (Hodgkinson *et al.*, 1993). The basic region of this non-growth-related protein was also replaced by the acidic extension, and this A-Mitf was analyzed for its ability to affect H-Ras and v-Myc cooperative transformation. As usual, a low level of focus formation was obtained with H-Ras alone, and this activity was increased three-fold by co-expression of v-Myc (**Figure 6.9**). Overexpression of wild-type Max does not affect the efficiency of transformation of H-Ras and v-Myc. However, D-Max-C inhibits transformation greater than 40%. The acidic Max protein (A-Max-C) is twice as effective as the Max basic region deletion (D-Max-C) in repressing cellular transformation. This indicates the strength of interaction between v-Myc and A-Max-C. Finally, A-Mitf overexpression is only minimally inhibitory to cellular transformation by H-Ras and v-Myc. This indicates the effect on focus formation by the modified Max proteins is specific and sensitive to the modifications that have been engineered into them.

Discussion

Utilization of dominant negative forms of transcription factors to study their importance *in vivo* is an effective means to analyze function. However, most dominant negatives analyzed to date must be overexpressed at very high levels to be biologically effective. For example, the ADD1 protein (adipocyte determination and differentiation-dependent factor 1) is a basic helix-loop-helix/leucine zipper protein that binds DNA as a homodimer and is involved in adipocyte development and cholesterol homeostasis. Kim and Spiegelman (1996) created a point mutation within the basic domain of ADD1 and tested this mutant ADD1 for DNA binding activity. The ADD1 protein with the point mutation within the basic region had completely lost its ability to bind DNA as a homodimer. This mutant ADD1 protein also abolished the DNA binding activity

of wild type ADD1 but only if expressed in 5-fold excess over wild type ADD1 (Kim and Spiegelman, 1996). While the use of dominant negative proteins is valuable to the study of transcription factor function, unless the dominant negative proteins are naturally-occurring or can be used at physiologically relevant levels within a cell, the effects observed may not provide accurate details of the situation *in vivo*.

The identification of B-ATF as a physiological dominant negative to AP-1 function is important since *B-ATF* exhibits a tissue-restricted pattern of expression (Dorsey *et al.*, 1995; Echlin *et al.*, in prep.; Johansen *et al.*, in prep.). EBV-positive B cell lines (Johansen *et al.*, in prep) and T cell lymphocyte cell lines (Hasegawa *et al.*, 1996) express *B-ATF*, and Northern blot analysis of human blood-forming tissues indicates that *B-ATF* expression is correlated with later stages of hematopoietic maturation (Echlin *et al.*, in prep). This is important since AP-1 activity is significant in hematopoietic cell lineages. For example NF-AT, a nuclear factor present in T cells and essential for induction of the interleukin-2 gene, consists of two components. One part of NF-AT pre-exists in T cells and is translocated to the cell nucleus upon stimulation; the other component of NF-AT is newly synthesized c-Fos and c-Jun proteins (Jain *et al.*, 1992). The presence of a naturally-occurring inhibitor to AP-1 in these cells could be important in the regulation of the interleukin-2 response of T cells. A possible role for B-ATF in cell maturation or differentiation is supported by analysis of the growth properties of cells that overexpress B-ATF protein. **Figure 6.2** shows that a cell line stably expressing B-ATF grows much slower than its parental cell line. Due to slowed growth, these cells never quite reach the saturation density of C3H10T1/2 cells by the end of the experimental counting period. Furthermore, colonies overexpressing B-ATF have altered size and morphology when compared to normal counterparts (**Figure 6.3**). These observations can also be explained as resulting from the slowed growth of B-ATF expressing cells. Finally, the ability of B-ATF to abrogate H-Ras-mediated transformation in two different cell lines (C3H10T1/2, **Figure 6.4**; and NIH 3T3, **Figure 6.5**) demonstrates the strength of the B-ATF dominant negative. Even in cells stimulated to grow and to become resistant to contact inhibition because of activated H-Ras, B-ATF still exhibits growth-suppressing properties. Thus, in a subset of cells which endogenously express B-ATF protein, AP-1 activity can be modulated, and cellular growth properties modified accordingly.

The design and use of the transcription factors with acidic extensions (A-Fos and A-Max, **Figure 6.6**) represents another mechanism to generate dominant negative factors *in vivo*. While these proteins are not naturally-occurring, they are very potent and are effective at low concentrations.

For example, in the case of A-Fos, a one molar equivalent of dominant negative A-Fos protein is sufficient to completely abolish DNA binding by wild-type Fos and Jun (Olive *et al.*, 1997). Investigation of the effects of A-Fos on cellular growth and transformation indicates that this engineered dominant negative protein can function in complex biological assays as predicted. Cellular transformation by the *H-ras* oncogene depends on Jun activity (Smeal *et al.*, 1991; Johnson *et al.*, 1996). While co-expression of Fos protein that is missing the TAD (B-Fos) does inhibit transformation of C3H10T1/2 cells (**Figure 6.7**), the greatest reduction of focus formation mediated by H-Ras occurs when the A-Fos (acidic Fos) dominant negative protein is co-expressed in cells. This protein is even more effective at repressing transformation by H-Ras than a Fos protein that is missing both the TAD and the basic region (compare the efficiencies of focus formation of H-Ras + 0-Fos [40%] to that of H-Ras + A-Fos [30%]; **Figure 6.7**). Interestingly, all of the dominant negative Fos proteins analyzed affect cellular transformation to a greater extent than cell growth (compare **Figure 6.7** to **Figure 6.8**), suggesting that these proteins may be useful in distinguishing the role of AP-1 in cellular transformation from its role as a general growth inducer.

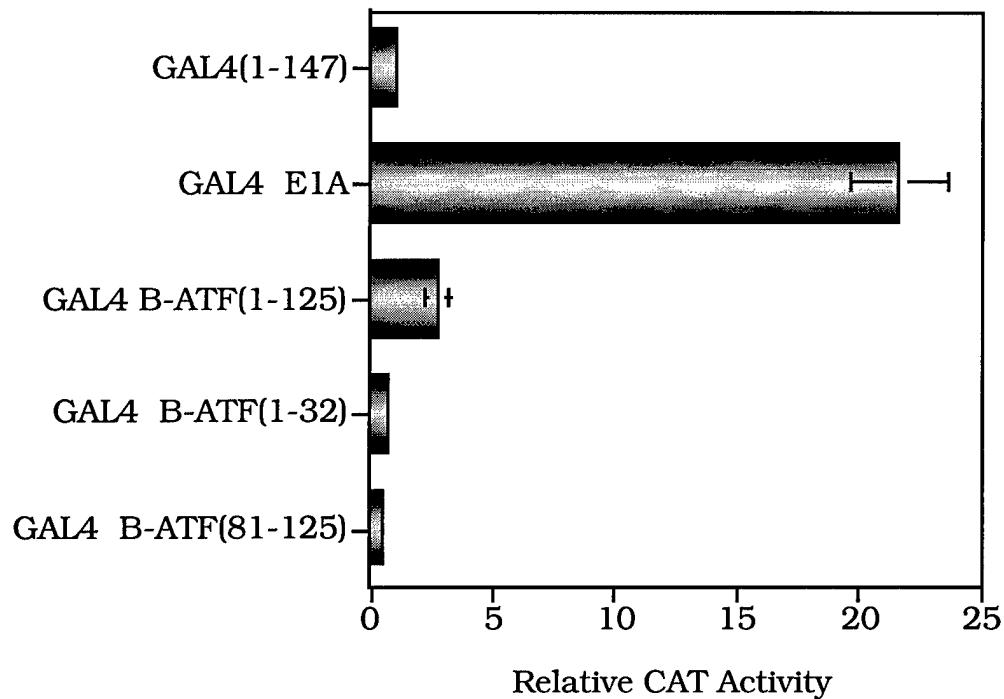


Figure 6.1 B-ATF does not contain a transcription activation domain. The full-length B-ATF protein or subregions of B-ATF were fused in-frame to the DNA binding domain of GAL4. C3H10T1/2 cells were transiently transfected with 5 μ g of the (GAL4)₅ E1B TATA CAT reporter, 5 μ g of the desired GAL4 fusion, and 5 μ g of RSV-LacZ to control for transfection efficiency. CAT activity was determined as described in Materials and Methods. The CAT activity of each sample is expressed as a fold increase over the activity of the GAL(1-147) vector alone, which is set at 1.0. Each experiment was performed three times and the results averaged. Error bars indicate the standard deviation in the experimental groups.

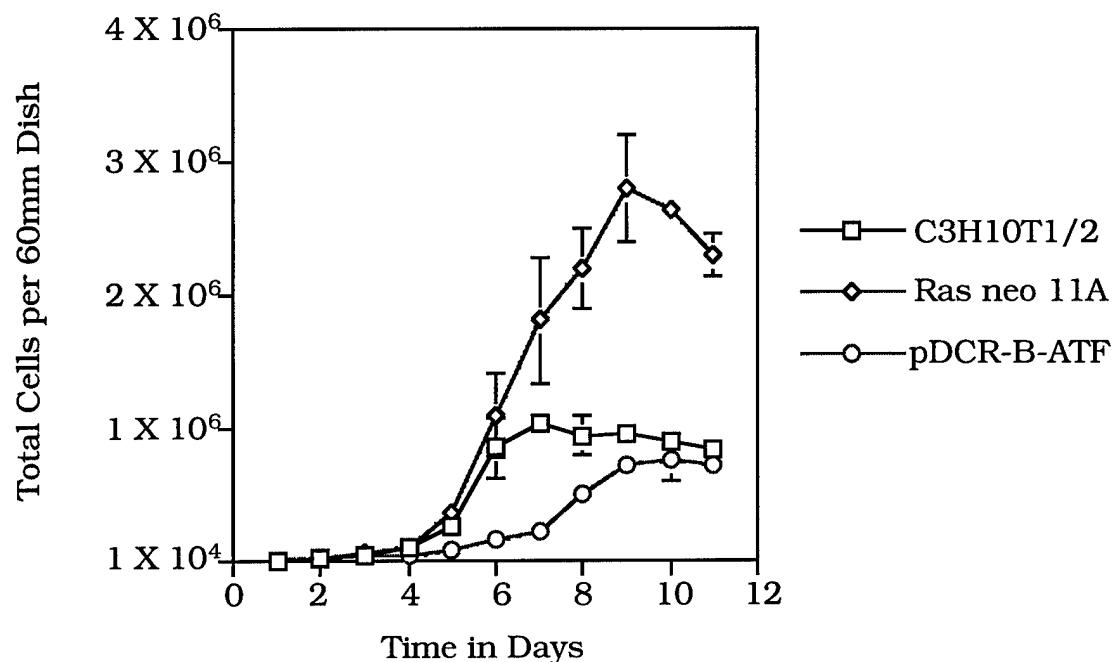


Figure 6.2 Growth properties of three fibroblast cell lines. The parental C3H10T1/2 cell line, Ras neo 11A cells (which overexpress activated human H-Ras; Weyman *et al.*, 1998), and pDCR-B-ATF (Echlin *et al.*, in prep) were seeded at 1×10^4 cells per 60 mm dish in growth medium (BME + 10%FBS + P/S). Cells were fed every three days, and the total cell number per plate was determined on 9 consecutive days. Total number of cells for each cell line is plotted over time for the duration of the counting period. Four values were averaged for each point, and the error bars indicate the variation obtained between experiments.

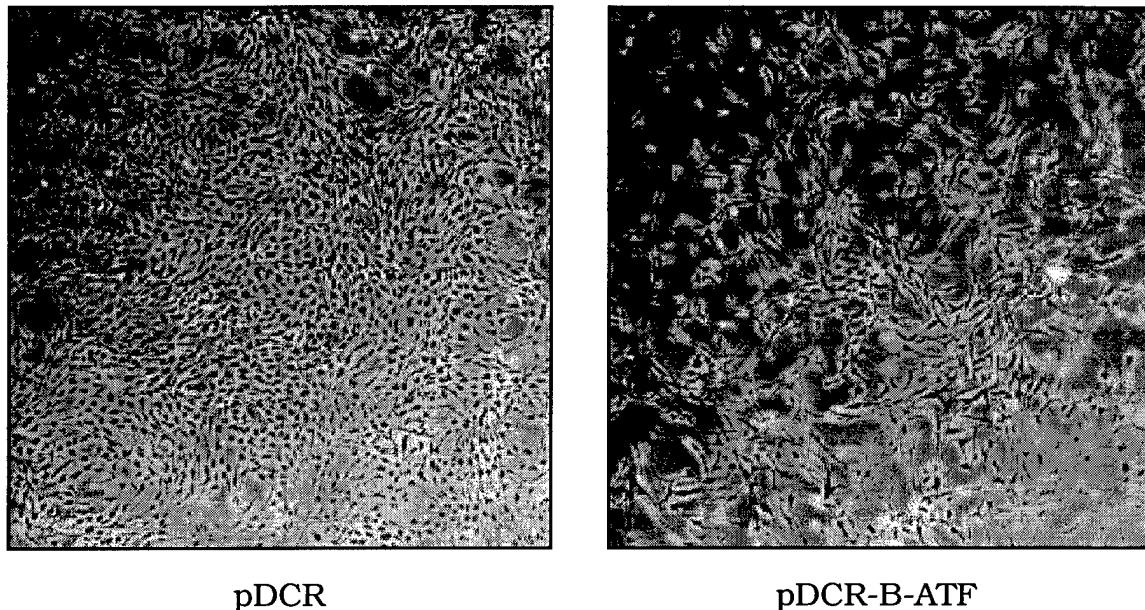


Figure 6.3 Organization and morphology of neomycin-resistant control colonies and colonies expressing B-ATF. Stable colonies generated following the transfection of C3H10T1/2 cells with 100 ng of pDCR control vector containing the gene conferring neomycin resistance or 100 ng of the pDCR-B-ATF plasmid which also encodes the full-length B-ATF protein were selected in G418-containing medium for 14 days. Photos taken under bright light conditions show the internal morphology of two similarly-sized colonies.

Groups	Number of Foci Per Experiment					Relative Focus Formation
	1	2	3	4	5	
H-Ras	148	132	98	184	141	100%
H-Ras + pDCR	152	147	---	168	109	96%
H-Ras + pNLVP16	---	---	---	202	157	110%
H-Ras + pDCR-B-ATF	53	85	29	119	84	51%
H-Ras + B-ATF-VP16-TAD	---	401	---	286	372	240%
pNLVP16	---	0	---	---	---	0%
pDCR-B-ATF	---	---	---	0	---	0%
B-ATF-VP16-TAD	---	0	---	0	---	0%

Figure 6.4 Transforming abilities of H-Ras in the presence or absence of overexpressed B-ATF proteins. C3H10T1/2 cells were stably transfected with 200 ng pT24 *H-ras*, *pNLVP16*, *pDCR-B-ATF*, or *B-ATF-VP16-TAD* or 200 ng pT24 *H-ras* plus 600 ng *pDCR*, *pNLVP16*, *pDCR-B-ATF*, or *B-ATF-VP16-TAD*. After 14 days of growth in reduced serum medium, cells were fixed, stained, and examined for cellular transformation as described in Materials and Methods. The number of foci formed by H-Ras is set at 100%, and the level of focus formation obtained in other groups is expressed as a percentage of that value.

Groups	Efficiency of Focus Formation
H-Ras	100%
H-Ras + pEMscribe-B-ATF	64%
pEMscribe-B-ATF	0%

Figure 6.5 Transforming abilities of H-Ras in the presence or absence of overexpressed B-ATF. NIH 3T3 cells were stably transfected with 200 ng pT24 *H-ras*, 200 ng pT24 *H-ras* and 600 ng *pEMscribe-B-ATF*, or 600 ng *pEMscribe-B-ATF*. After 14 days of growth in reduced serum medium, the cells were fixed, stained, and examined for cellular transformation as described in Materials and Methods. The number of foci formed by H-Ras is set at 100%, and the level of focus formation obtained in other groups is expressed as a percentage of that value. The efficiency of focus formation value was determined by averaging the number of foci obtained from four independent experiments.

RERNKMA AAKCRNR RRELTDT LQ basic (B) Fos
LARENNEE LEKEAEE LEQELAE LE acidic (A) Fos

RAHHNALERKRRDH[↑]IKDSF basic (B) Max
DPDEEDDEEELEELED[↑]SF acidic (A) Max

Figure 6.6 Sequences of the wild-type and engineered proteins used as dominant negatives to examine the role of certain transcription factors in cell growth and transformation. The basic regions of human c-Fos (top group) and mouse Max (lower group) were removed and replaced by a stretch of acidic amino acids (Olive *et al.*, 1997; Krylov *et al.*, 1997). The arrow in the top group points to the “L” representing the first leucine in the Fos leucine zipper. The arrow in the bottom group points to the “F” representing the phenylalanine in helix 1 of the Max helix-loop-helix.

Group	Number of Foci per Experiment				Efficiency of Focus Formation
	1	2	3	4	
H-Ras	207	134	123	156	100%
H-Ras + B-Fos	---	---	86	80	59%
H-Ras + O-Fos	75	54	---	42	40%
H-Ras + A-Fos	53	41	---	35	30%
H-Ras + A-CREB	---	97	---	---	72%

Figure 6.7 Transforming abilities of activated H-Ras in the presence of truncated (B-) or modified (O- and A-) Fos proteins. C3H10T1/2 cells were stably transfected with 200 ng of pT24 *H-ras* or *H-ras* and 600 ng of *B-fos*, *O-fos*, *A-fos*, or *A-CREB*. After 14 days of growth in reduced serum medium, cells were fixed, stained, and examined for cellular transformation as described in Materials and Methods. The number of foci formed by H-Ras is set at 100%, and the level of focus formation obtained in other groups is expressed as a percentage of that value.

Group	Number of Colonies per Experiment		Efficiency of Colony Formation
	1	2	
H-Ras	427	471	100%
H-Ras + B-Fos	---	349	78%
H-Ras + O-Fos	288	233	58%
H-Ras + A-Fos	314	249	63%

Figure 6.8 Effect of co-expressing of H-Ras and various dominant negative Fos proteins on cellular growth. To assess colony formation, 1/6 of the cell suspension generated in **Figure 6.7** to analyze focus formation was further diluted 1:6 and maintained in BME + 10%FBS + P/S + 400 µg/ml G418. After 14 days of growth in selective medium, colonies were fixed, stained, and counted as described in Materials and Methods. The amount of colonies formed by H-Ras is set at 100%, and the efficiency of colony formation obtained in other groups is expressed as a percentage of that value.

Group	Efficiency of Focus Formation
H-Ras	34%
H-Ras + v-Myc	100%
H-Ras + v-Myc + wt Max	96%
H-Ras + v-Myc + ΔMax-C	56%
H-Ras + v-Myc + A-Max-C	28%
H-Ras + v-Myc + A-Mitf	77%

Figure 6.9 Transforming abilities of activated H-Ras and v-Myc in the presence of wild-type or modified Max proteins. C3H10T1/2 cells were stably transfected with 200 ng pT24 *H-ras*, 600 ng pMC29 *v-myc*, and 2 µg of each *Max* or *Mitf* plasmid. After 14 days of growth in reduced serum medium, cells were fixed, stained, and examined for cellular transformation as described in Materials and methods. The number of foci formed by H-Ras and v-Myc is set at 100%, and the level of focus formation obtained in other groups is expressed as a percentage of that value. The efficiency of focus formation value is calculated from the number of foci obtained from three independent experiments.

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

The Myc proteins are key regulators of cellular proliferation, differentiation, transformation, and apoptosis. While these functions of the Myc protein rely upon an intact dimerization and DNA binding domain (bHLH/LZ) in the carboxy terminus of Myc, the integrity of the amino terminus of Myc is also critical for these Myc-mediated activities. Mutational analysis of the amino terminal transcription activation domain (TAD) of v-Myc suggests that residues mapping to amino acids 1-112 of the terminus of v-Myc are very potent activators of transcription.

Interestingly, these GAL4-v-Myc fusion proteins are missing the highly conserved MHR II domain. This parallels other studies showing that sequences comprising MHR II are dispensable for transcriptional activation (Brough *et al.*, 1995). It also indirectly suggests that MHR II could play a negative role in transcriptional activation of Myc.

Additionally, 5' mutations engineered into the v-Myc TAD were informative as they uncovered a potential negative regulatory region mapping to residues 220-245 of the v-Myc TAD. Since both protein stability and DNA binding capacity were not affected by these deletions, the discrepancies in transcriptional activity of Myc proteins that terminate at v-Myc residues 219 versus 244 reflects an inherent difference in transactivation potential which is mediated by the presence of these 25 amino acids. Deletion of v-Myc residues 220-245 from full-length v-Myc results in a protein that is a stronger transactivator and more efficient transforming protein than wild-type v-Myc. These observations are in agreement with other studies which analyzed a similar mutation in the v-Myc TAD. Work by Heaney *et al.* (1986) investigated the ability of v-Myc to transform primary avian embryo macrophages and fibroblasts when a similar mutation was generated in v-Myc. While this v-Myc mutant remained efficient in its ability to transform chicken fibroblasts, the ability to transform avian macrophages was abolished. These observations indicate the importance of this small region of v-Myc to both transcriptional activation and cellular transformation. **Figure 1.2** shows that within this short stretch of amino acids is an *in vivo* casein kinase II (CKII) phosphorylation site (Luscher *et al.*, 1989) and an extremely acidic group of amino acids. Mutation of the CKII phosphorylation site does not affect Myc activity (S. Hann, personal communication). To date, no published results using Myc proteins altered in this acidic domain have been reported. To thoroughly test this tiny yet obviously important region of v-Myc, mutation of this acidic region to an uncharged stretch of residues would provide a reagent suitable to investigate whether or not the acidic nature of these 25 amino acids mediates the repressive effect on Myc transactivity. In addition to mutation of the acidic domain, a cell line stably expressing v-Myc(Δ 220-245) would be valuable to

investigate the effect of this Myc mutation on both cell growth and Myc-induced apoptosis as compared to the Myc neo 13A cell line which overexpresses wild-type v-Myc.

The identification of OS-9 as a specific Myc amino terminal-interacting protein is significant and intriguing at the same time. The amplification of the *OS-9* gene in two-thirds of human osteosarcoma cell lines (Su *et al.*, 1996) indicates that elevated expression of OS-9 may play a role in tumorigenesis. The frequency with which the *OS-9* cDNA was isolated in the yeast two-hybrid screen (8 of 22 independent, positive clones) using v-Myc MHR II as a bait suggests this interaction was not simply an artifact of the system or of the library used. In addition to detecting interaction between Myc and OS-9 in yeast, *in vitro* binding assays using radiolabeled OS-9(424-667) protein and bacterially-expressed fusions of GST and v-Myc demonstrate that the OS-9/v-Myc association relies on the presence of MHR II within Myc. While direct immunoprecipitation of OS-9 and v-Myc was unsuccessful from transiently transfected cells, an indirect *in vivo* association was confirmed using a modified version of the two-hybrid system in mammalian cells. This phenomenon is reminiscent of what was observed between the amino terminus of Myc and the retinoblastoma protein, Rb. The TAD of c-Myc was initially shown to specifically interact with Rb via GST pull-down experiments (Rustgi *et al.*, 1991). Unfortunately, co-immunoprecipitation experiments failed to demonstrate the *in vivo* interaction between these two important cell cycle regulators. Interestingly, a recent study demonstrates an interaction between the amino terminus of v-Myc and Rb using the same modified mammalian two-hybrid system. In these experiments, a 4.5-fold increase in Myc-mediated transcriptional activation was detected in the presence of DNA-bound Rb (Adnane and Robbins, 1995), which is similar to the 2.5-fold increase in transactivation due to the interaction of Myc and OS-9.

The predominantly cytoplasmic localization of full-length OS-9 is problematic. Since Myc is a nuclear protein, a spatial discrepancy exists between the localization of Myc and OS-9. However, truncation of OS-9 allows most of the exogenous OS-9 protein to be translocated to the nucleus. This suggests several things: (1) that the SV40-type nuclear localization signal (NLS) within the carboxy terminus of OS-9 is functional and efficiently directs truncated OS-9 to the nucleus, (2) that the amino terminus of OS-9 must somehow mask the effects of the NLS or at least suppress nuclear localization well enough to escape detection by immunofluorescence, (3) that retention of an NLS in OS-9 may allow nuclear localization of the protein under some cellular circumstances. At the present time, the regulation of OS-9 intracellular location by cell cycle or proliferation events has not been investigated.

Due to the dramatic inhibitory effect of OS-9 on Myc transcriptional activity despite the observed subcellular location of OS-9, two mutations were made in the protein. One of these mutations deletes the NLS of OS-9 in the context of the full-length protein (OS-9 Δ NLS). The other mutation retains the NLS plus an additional 12 C-terminal amino acids of OS-9 before a frameshift causes premature termination of the protein (OS-9-STOP). The activity of these OS-9 mutants towards Myc transcription activation then was tested. **Figure 7.1** indicates that while the full-length OS-9 protein is an efficient inhibitor of Myc transcriptional activity, the OS-9 Δ NLS protein has a reduced capacity to repress Myc transcriptional activation, presumably due to its exclusive cytoplasmic location. Perhaps even more interesting is the continued repressive activity of OS-9-STOP as compared to wild-type OS-9 (D.R. Echlin and E.J. Taparowsky, unpublished observations). These data suggest the 12 residues 3' to the NLS may mediate the Myc/OS-9 interaction. Immunofluorescence to determine the precise location of these mutant OS-9 proteins will be performed. If these proteins are found in the expected cellular compartments (OS-9 Δ NLS in the cytoplasm and OS-9-STOP in the nucleus), then the effects of these mutant OS-9 proteins will be further analyzed by others in the laboratory.

The inability of OS-9 to impact Myc-mediated cellular transformation was disappointing. Due to the documented role of MHR II in induction of transformation by Myc, a protein found to interact with this region of Myc would be a likely candidate for a modulator of Myc-mediated transformation. While no effect on Ras- or Ras plus Myc-induced cellular transformation was observed in either the murine fibroblast cell line C3H10T1/2 or in the Rat 1a fibroblast cell line (Echlin and Taparowsky, data not shown), the ability of OS-9 to modulate v-Myc-mediated transformation of avian cells has not been examined. In addition, we have not examined the levels of endogenous OS-9 protein within C3H10T1/2, Rat 1a, or chicken embryo fibroblasts, and it is possible that levels are sufficient for transformation events.

The ability of OS-9 to impact transcriptional activation by both Myc and the viral protein VP16 suggests a more “global” effect of OS-9 function. In agreement with this observation, OS-9 can repress transcription of all activators when bound to DNA. This suggests OS-9 functions directly on promoters. Dimerization between Myc and Max and subsequent binding to E box sequences within the promoters of Myc-responsive genes activates transcription of those genes. OS-9 can interact with the DNA-bound Myc/Max complex as shown by the ability of OS-9 to repress transactivation of the (MBS)₃ TK CAT reporter, which is regulated by three Myc E box binding sites. *In vivo*, the interaction of OS-9 with the Myc/Max heterodimer would lead to OS-9 being positioned in the proximity of the promoter. At this site, OS-9 could interact with factors

of the general transcription machinery and influence transcriptional activation. Since OS-9 inhibits the transactivation potential of all activators when tethered to DNA, OS-9 is most likely recruiting a repressor protein to the promoter. This is similar to the situation involving the Max-binding partner Mad, whose expression is induced upon cellular differentiation (Ayer *et al.*, 1993). The Max/Mad heterodimers bind to Myc E box DNA and repress genes normally activated by Myc/Max complexes. Mad has recently been shown to interact with the mammalian homolog of the yeast Sin3 repressor, mSin3 (Ayer *et al.*, 1995). The Max/Mad complex is believed to repress transcription by tethering the mSin3 repressor to DNA. A similar scenario could explain the transcriptional repression of Myc by OS-9.

Enhancement of Myc-mediated repression by OS-9 was an unexpected observation and was confirmed using both a viral (adenovirus-2 major late) and a cellular (adrenomedullin) promoter system. A possible mechanism for repression by Myc is based on the interaction discovered between Myc and the general transcription factor TFII-I. TFII-I activates promoters through Inr sequences, suggesting that association with Myc may preclude activation by TFII-I at the Inr (Roy *et al.*, 1993). The interaction between Myc and TFII-I is dependent upon an intact HLH/LZ domain in the Myc carboxy terminus, which leaves the amino terminus of Myc free to interact with different factors such as OS-9. Perhaps OS-9 stabilizes the Myc/TFII-I complex or results in a greater affinity of Myc for TFII-I, each of which could account for the increased repression of Inr-containing promoters observed.

Finally, perhaps the most provocative data generated suggest that OS-9 modulates the effect of Myc expression on cellular growth and apoptosis. Overexpression of Myc recently has been demonstrated to induce expression and accumulation of the cell cycle regulators cyclin A and E. This induction leads to the association of cyclin E and the cyclin-dependent kinase cdk2 (Jansen-Durr *et al.*, 1993) which in turn serves to phosphorylate the Rb protein. In its hypophosphorylated form, Rb binds to the transcription factor E2F and keeps it in a functionally inactive state. However, when Rb is phosphorylated by cyclinE/cdk2, the E2F transcription factor is released and is free to transcriptionally activate genes required for cell cycle progression (Weinberg, 1995). Repression of Myc activity by OS-9 could interfere with the process of cyclin E and A accumulation, which could explain the slowed growth of cells overexpressing v-Myc and OS-9. As for the role of OS-9 in inhibiting apoptosis, this could be linked to the activity of another Myc target gene, *ODC*. Activation of *ODC* by Myc could result in the dangerous accumulation of reactive oxygen species known to play a role in cellular apoptosis. If any of the genes encoding detoxifying enzymes have been mutated, this could prove to be a hazardous

predicament for the affected cell. However, in cells that have sustained amplification of the *OS-9* gene, cell death could be averted by the suppression of Myc transactivation and, indirectly, Myc-mediated apoptosis. Repression of this activity by *OS-9* would help to ensure survival of the growing tumor. **Figure 7.2** depicts this proposed model of the functional consequence of *OS-9*/Myc interaction.

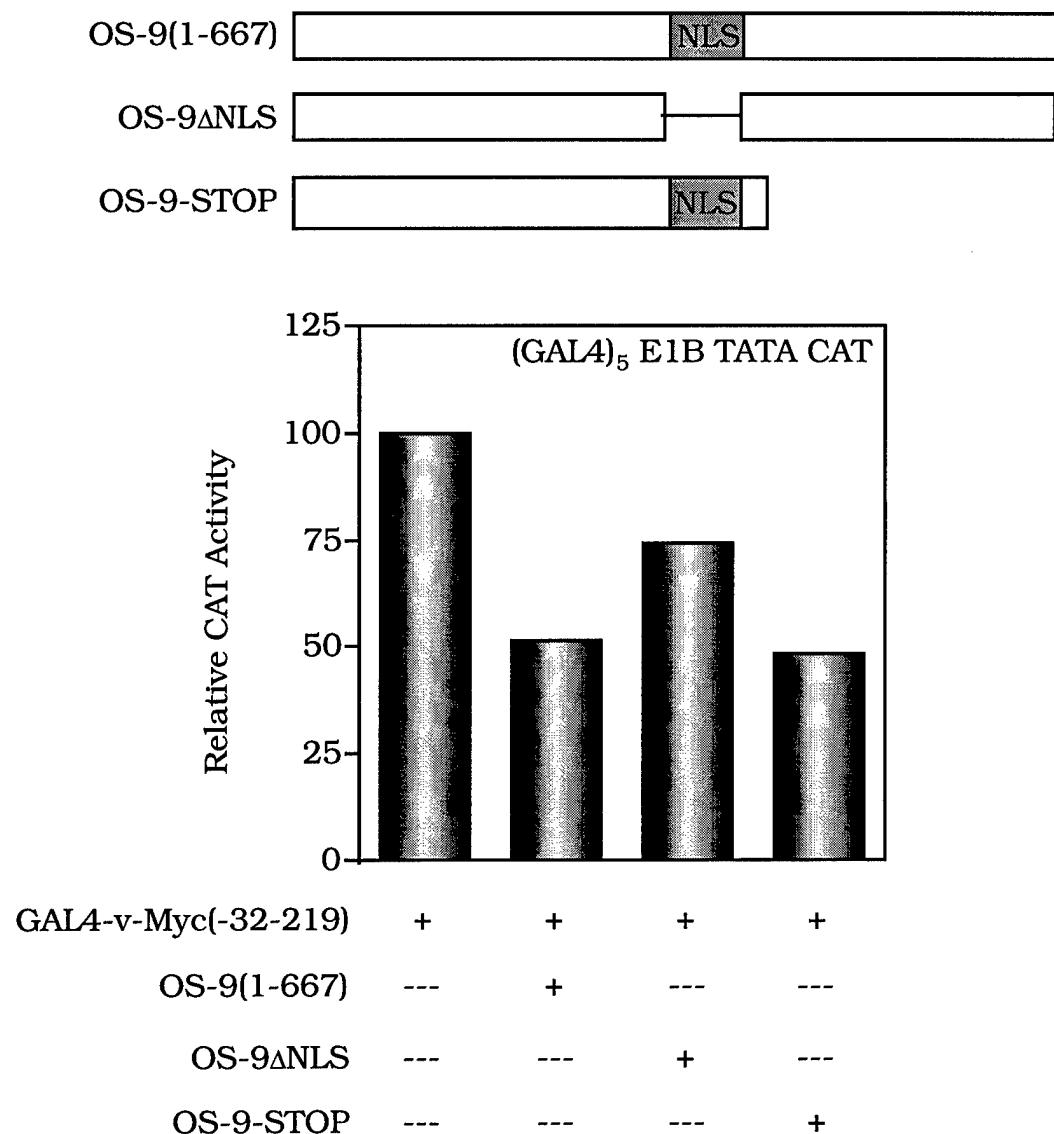


Figure 7.1 Influence of full-length and mutant OS-9 proteins on transcription activation by Myc. C3H10T1/2 cells were transiently transfected with 5 μ g each of the (GAL4)₅ E1B TATA CAT reporter and GAL4-v-Myc(-32-219) activator and a 2-fold molar excess of OS-9 plasmid DNA. CAT activity was determined as described in Materials and Methods. The activity of GAL4-v-Myc(-32-219) in the presence of control vector is set at 100%, with the activities measured in other samples expressed as a percentage of that value. This experiment was only performed once.

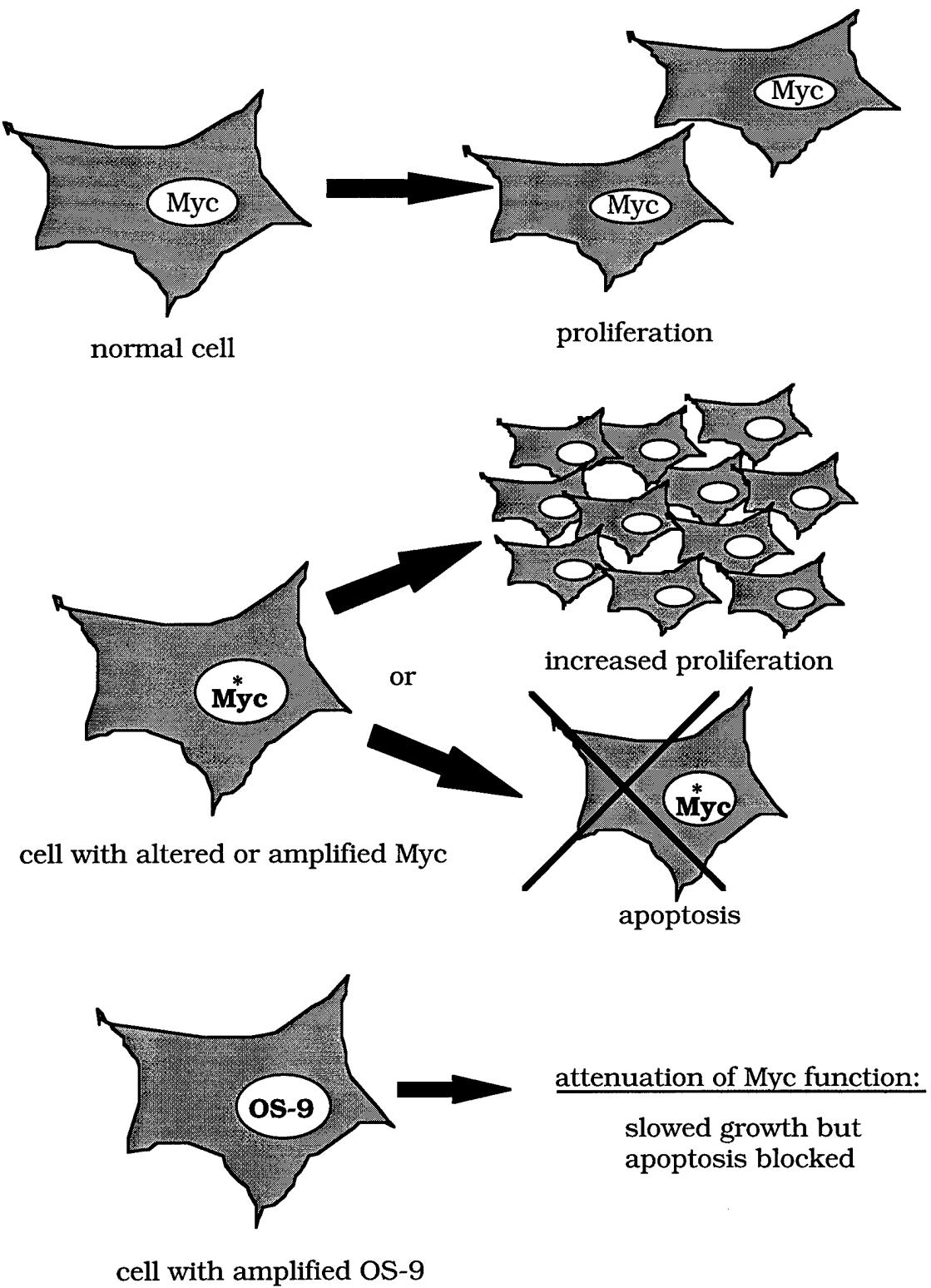


Figure 5.2 Model of the functional interaction between Myc and OS-9.

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